FOOD AND DRUG ADMINISTRATION
Toxicology Study Selection and Review Committee
(TSSRC)

Meeting of:

Toxicology Study Selection and Review Committee

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P R O C E E D I N G S (9:00 a.m.)

Agenda Item: Introduction

DR. DA COSTA: Good morning everyone. Welcome to the fall 2019 TSSRC. It is good to see you all here. Before I forget because it is not in the agenda, tomorrow we are going to have a small satellite meeting that is going to be dealing with air-liquid interface system that (indiscernible name) is going to be talking about. We will have colleagues from NIOSH, the other core agency of the National Toxicology Program other than NIEHS and FDA joining us this afternoon and for the meeting tomorrow. You are all invited to join us here tomorrow if you are interested in that meeting. We are going to be meeting between 9 and 11 a.m. We do not really have a structure or a defined agenda because we just want to allow for free discussion of the interests. NIOSH is going to be making a presentation on the work.

At this stage, I will ask Nigel if he has any words to offer.

DR. WALKER: Good morning everybody and those online. There is quite a bit of folks. Just thanks for everyone spending the time to come and review things that are currently ongoing in the interagency agreement. This has been longstanding now, 28 years or so. This is really important. It is really important to get the feedback on

the value of the different projects, how it impacts on regulatory decision making because we take that under advisement when making decisions about what is going to go forward. This is really valuable information that we receive. Your attention is really appreciated.

DR. SLIKKER: I just want to welcome everybody and especially our colleagues from NTP, NIEHS. It is always great to meet and to meet with all the other center representatives that are here. I appreciate your time coming to look at the data that everyone is generating for FDA and to move the FDA forward. We appreciate everybody being here together here today. Thank you.

DR. DA COSTA: Thank you. Perhaps we can start doing the introductions in the room. Why don't we start on the table? Bob?

(Introductions)

DR. DA COSTA: Thanks everyone. And I see that we also have a number of folks online. Thank you also for connecting. As always, one of the exciting moments in this meeting is recognizing that you were sent the transcripts from the last meeting, which you always carefully read and provide comments. If you have any comments then be sure to provide them to Amy or me.

Very important for those of you on the phone, I would really appreciate if you muted your phones during the

talks and then following the talks in the question and answer section then you can unmute should you want to ask a question.

We need to schedule these meetings well in advance because unless you book these meetings at least - meeting room at least in advance, we will lose the possibility of even getting it booked. We already have the next meetings booked. I would appreciate if you were to enter those dates in your calendars to make sure that the dates are available for you to attend the TSSRC.

DR. WALKER: A key thing to note - November 5 to 6, 2020 actually is a Thursday and Friday. I know we normally do Wednesday/Thursday meetings, but it may not be lost on you that November 3 is Election Day that week so we wanted to avoid having folks traveling on that Tuesday. That is why we are having it on the Thursday and Friday so folks can travel on the Wednesday, which is what we did last time and it was chaotic.

DR. DA COSTA: A lot of people were not happy about our previous scheduling on Election Day.

Just going very briefly as Nigel already mentioned, we are going on the 28th anniversary of our interagency agreement. And the objective of the agreement was the connection of toxicology and mechanistic studies on

FDA-regulated agencies that are nominated to NTP to be conducted at NCTR.

And the studies are designed to provide the FDA and other regulatory agencies. I will highlight that because Japanese authorities. The Chinese authorities. A lot of people use that and it stems from studies that are designed, discussed, and put together in this room, but to provide those agencies with identification and those response that to support risk assessments and risk management decisions.

There have been some changes on all these interagency agreements. It is structured and implemented. And Nigel will be talking a little bit more about these this afternoon. But an element that needs to be considered at this stage is that these studies for the studies to be approved for conduction under the IAA, they need to be of strategic interest to the NTP as determined by the very internal government, the scientific and government structure. Nigel will be chatting a little bit more about this.

Again, briefly, this particular meeting, the Toxicology Study Selection and Review Committee is charged with the oversight of studies on the interagency agreement. We meet twice annually. We conduct the review of protocol design on selection study progress.

This is a closed meeting. One of the great advantages of these meetings is that we can allow for direct dialogue between the product centers, NIEHS, FDA, and any other peers that we invite to the meeting in an open fashion. But what that means is that we are discussing preliminary - it has not been audited, et cetera. That should really not be shared outside of this meeting without prior concurrence from Nigel and me.

All of these works in a very streamline fashion because Amy Babb puts a lot of effort on putting this meeting together. Again, it starts 18 months before the meeting actually is convened. I am thanking Amy once again for all the work, the excellent work that she does.

We have booked a table at Olazzo, which is a restaurant where we have been a number of times. We find that it is a pretty good restaurant. You are all invited to join us. If you want to join us, I would just appreciate if you could let Amy and I know so that we can make sure that the table is large enough for you to join us. But you are certainly welcome to join us. They have good food and pretty good selection. We have a private sommelier, who handles the wine orders for us.

Luisa Camacho is currently conducting an IARC review in Leone so she could not be here. But she asked me if I could provide the very brief assessment of the status

of her study on lumbrokinase. The title of the study is assessment of the effects of a 28-day oral exposure to the fibrinolytic enzymes nattokinase and lumbrokinase individually or in combination with aspirin in adult Sprague-Dawley rates. This was a study that was nominated by the CFSAN.

The intent of the study was to try to ascertain whether there was an interaction between the effects of aspirin and lumbrokinase or nattokinase that could raise adverse effects.

The status of the protocol is that the data collection has been completed. The statistical analysis is nearly completed. Luisa is currently preparing manuscripts to report the data.

The main findings of the study were no evidence for increased risk of bleeding of one oral exposure to nattokinase or lumbrokinase under the conditions tested. Minimal evidence for interaction between nattokinase and aspirin. There were statistical differences observed only in a few hemostasis parameters and only in males. There was no evidence for interaction between lumbrokinase and aspirin, which I suppose is good news.

Just for a final update, I am also responsible for a study that is now being completed and titled

evaluation of brominated vegetable oil in Sprague-Dawley rats. This was also a study nominated by CFSAN.

The primary objectives of the protocol were to assess that response of a 90-day dietary exposure to BVO in Sprague-Dawley rats. This was on arm of the study and the other arm objective of the study was to evaluate the bioaccumulation and clearance of inorganic and organic bromine in organs and tissues of Sprague-Dawley rats upon dietary exposure to BVO.

The experimental procedures have been finalized. We currently have ongoing statistical analysis of the data from the two arms of the study. The manuscript of analytics is being finalized and this is a manuscript that I need to publish because it describes the analytical procedures, which then will be followed by the manuscript describing the toxicological findings.

The main outcomes of the study were that there was evidence of follicular cell hypertrophy in the thyroid. There was a reduction in serum T4 and increase in serum TSH and bromide. There was clear bioaccumulation of brominated fatty acids in multiple organs and fortunately an indication that the effects are reversible upon cessation of exposure.

That is all the updates that we have for today. I do not know if there are any questions about these updates.

DR. ZHOU: I was just wondering. Is it a total T4 or free T4.

DR. DA COSTA: That is a good question. T3 was not changed. But that is a good question. I would have to check that out. It has been a while that that data was generated.

DR. HANIG: So many years ago, I was involved - it was kind of like a summer job in a chemical company that made brominated peanut oil and so on. And one of the things that really was surprising was that when you opened up the can, it was a liberation of bromine that was coming out of the - did you ever notice anything like that in any of your studies?

DR. DA COSTA: No. I actually know that they use the last amount of bromine to the reaction because there is still residual double bonds have not been brominated.

DR. HANIG: That is the answer. Thanks very much.

DR. DA COSTA: I think that we are ready to go to our first presentation of the day, which is going to be Dan Doerge on giving what I believe is a final update on the studies of the pharmacokinetics of arsenic. Thank you.

Agenda Item: Arsenic: Pharmacokinetics, Update

DR. DOERGE: Good morning. To paraphrase one of the more famous Little Rock Natives, I am here to talk about our old friend, arsenic. As Douglas MacArthur said, food contaminants never die. They just fade away until they

don't. The issues of arsenic as a food contaminant are not to be belabored here. The point is despite the fact that it will not ever go away because it is ubiquitous in many common foods that people eat all over the world. The reason is flares up from time to time is that not only is arsenic acutely toxic, but it is involved in the etiology of many chronic diseases, most notably cancer and some other organ-specific toxicities.

We have been working on this for a while now since 2015 particularly on the links with perinatal exposures in a number of diseases, given our experience in a number of previous studies that examine this particular factor in risk assessment.

Again, human exposures do not really change a lot. The only thing that changes are when different food categories are identified and quantified that the risk assessment changes. Again, the data in food are not great. They are getting better all the time. The human exposure levels are pretty well understood - less than a microgram per kilogram body weight per day across the world even in cultures in East Asia where rice and seafood intake is much higher than it is in the US or other developed economies. The overall intake is pretty well understood and the risk assessment of arsenic has been problematic for years for risk assessment agencies all over the world.

The biggest problem has - the reason that the cancer aspects of arsenic are the ones that have been used the most frequently in risk assessment if not exclusively is that the studies that are available, it is the most sensitive endpoint in terms of lifetime exposures.

The interesting thing is that so many of these risk assessments have used human observations since the old days. There was not a good animal model for arsenic carcinogenesis.

The biggest problem with all these risk assessments and one I do not think people have taken on board so much is that the effect levels from the epidemiology studies are very similar to human dietary intake assessments from food. That is, there is margin of exposure of one when normally we look to 100 or 1000 or what the hell ever to be deemed safe.

The bigger problem with arsenic though is the strong body of epidemiological evidence on the impact of early life exposures for the etiology of cancer from this Chilean cohort that shows really large increases in incidences and decreases in latency for a number of tumors in a Chilean cohort that was exposed uniquely to high levels of arsenic early in life, that is, from birth to death in a well-defined exposure scenario that really

emphasized the importance of these early life exposures in cancer, probably other diseases as well.

The studies that we have been working on now for a few years are to understand the mechanisms in an animal model for human cancer and specifically these studies that we have done that involve measurements in the mouse are testing the hypotheses that metabolic and/or physiological immature in the young lead to elevated internal exposures to toxic arsenic species that then are involved in the second aspect in that interfering with developmental programming by these reactive species then take on increased importance due to the unique molecular targets presented during the process of development. And that either these metabolic immaturity or the developmental programming interruption - could be involved in the additional susceptibility that is manifested in these epidemiological studies from the Chilean cohorts that lead to increase incidences and decreased latencies of cancer and probably other diseases later in life.

There was some information known about the metabolism and disposition of arsenic species that sort of laid a framework, but kind of like when we started with BPA. I quipped that it had been studied to death. The problem was there was a lot there, but it did not - a lot of the key elements really had not been worked out. And if

that is what we tried to do is to take these studies and focus in on the most important elements of the metabolism disposition to understand more fully all the ramifications of these arsenic exposures in different modalities be it food or water and try to understand how they all fit together to inform the risk assessment and try to identify some opportunities to go a little further.

This slide encapsulates a lot. I am not going to go through it. The point is that the inorganic arsenic is extensively methylated very quickly upon entry into the body to form as the predominant species, the dimethylarsinic shown at the bottom here, all the way at the bottom, represents 98, 99 percent of AUC in the blood. This is a very important process.

In addition, our work that I will describe in a little bit more detail, not a lot, is that these intermediates like the monomethyl, which is a bound enzymatic intermediate that apparently small amounts can be released and to form a monomethyl derivative that is also present in blood and tissues to some extent.

But in addition, you will see all these double-headed arrows with various reductants including glutathione, as we will see later, can reduce these species both the MMA and DMA to these reactive and that is why the brackets are here. These are highly reactive species that

do not last long on their own that can then bind with proteins specifically thiol groups in proteins and because thiol groups are used so extensively in cell signaling processes that disruption of these processes by covalent modification with arsenic species disrupts the cellular regulatory processes involved in not only mature cells, but also in developing cells. This is the chemical manifestation of our susceptibility hypothesis.

And what I am going to do is talk about the publications that have resulted and highlight some of the important elements for the risk assessment. In the beginning, we did a pilot study where we looked at a large number of tissues and blood to try to get an overview and focusing also on the excretion. Again, I cannot emphasize the importance of the complete methylation - well, not complete, but for these purposes, virtually complete formation of dimethylarsinic, which is cleared from blood and tissues. It is unlike lead, unlike mercury where it goes in and stays in deep compartments like bone or wherever because arsenic is excreted as DMA(V) extensively in urine.

One of the important things we discovered in this first study was that the higher you go in dose probably the less relevant it is for risk assessment because of pharmacokinetic non-linearity that leads to excessive

amounts of the intermediates and the reactive substrate of inorganic arsenic. That in a normal situation of low dose, you would be dealing with virtually all dimethylated arsenic so that high dose is an artifact-inducing regime.

For our studies then, we wanted to go as low as possible to maintain the pharmacokinetic linearity where you understood the importance of cellular thiol binding of the trivalent arsenic species, all three of them. And identified a surrogate tissue where would do pharmacokinetic measurements that relate to tissues' concentrations, but are much easier to do experimentally.

We then followed that up with a more detailed pharmacokinetic study where we, for the first time, identified the importance of first-pass metabolism in the processing of inorganic arsenic and other species.

A key finding there was that the levels of the substrate arsenite and the intermediate monomethylarsonate are highest in the GI tract, which should not be a surprise. But this really has some important implications in that the reactant species are highest, not only because it is the first place where the body gets to interact with inorganic arsenic, but then this is also the host tissue for the microbiome and the communication between immune cells in the GI tract with the microbiota are really important in determining the healthy interaction with

microbiome as well as the ability of the bacteria in the gut to process and be affected by inorganic arsenic as well so that - it is pretty clear that contaminants like this can have important effects on the microbiome. But I think these studies really gave a clear indication because the levels are the highest in the GI tract.

The next first pass after portal blood delivery is the liver where the levels of inorganic arsenic and monomethyl bound are lower than the GI tract, but higher than most of the rest of the tissues, which we then quantify with measurements and even identified some tissues like the brain muscle that have the lowest levels of all the - indication of the lowest internal exposures of all tissues to the reactive trivalent arsenic species, including the primary reactant species, the DMA(III).

We followed up these results then in pregnant mice and made measurements both in the dams and the fetuses this time using repeated dosing, focusing on our so-called low dose to minimize non-linearities and made extensive measurements in the dams and fetuses, showed that the metabolism in the dam was really quite effective in minimizing the exposure to the fetus particularly for the MMA and inorganic arsenic, which is not observed in the fetus at all so that the maternal metabolism minimizes this toxicity potential.

However, there was evidence for DMA(III) binding albeit at levels that were much lower than those in the maternal tissues. Nonetheless, the binding of DMA within brain and all other tissues is evidenced for metabolic activation in all these compartments.

We then followed this up with a study in neonatal mice and made the same kinds of measurements and compared pharmacokinetic parameters from blood and tissues and found that for the reactor species that the arsenite, MMA(III) and DMA(III) all showed a pattern of deficient metabolism in the neonatal - particularly the youngest, but it developed throughout neonatal life so that by weening the pups were very similar to the adults in their capacity to metabolize and excrete arsenic species.

The differences in blood were consistent with deficiencies in the excretory function for DMA(V) and the increased binding in the tissues correspondingly provided evidence for an increased susceptibility due to deficiencies in pharmacokinetics in neonatal mice.

We further made extensive measurements in blood and milk to show that there is very limited lactational transfer of any arsenic species in the mouse model and that this confirms findings recorded from human breast milk.

This is actually a fairly important point because the prototype model, animal model for arsenic

carcinogenesis that was a breakthrough reported out by the NIEHS a few years ago. In their studies, they assumed efficient lactational transfer in their whole life exposure model, but unfortunately, that did not prove to be an accurate assumption. As a result, it is probably likely that the dose responses observed in that model will probably skew to the high side because they did not get exposure during this critical neonatal window.

Again, I have emphasized the importance of dimethylarsinic in inorganic arsenic toxicity because it is the predominant metabolite in blood and tissues.

We did a complementary study, used dosing with dimethylarsinic. Like inorganic arsenic, it is extensively metabolized, high bioavailability. But what we found was that the levels of bound DMA were very similar whether we dose with inorganic arsenic or with DMA. And what that shows is that there is extensive reductive metabolism like I showed in that scheme. Again, these tissue levels varied depending on the site in the transit through the body so very high levels in the intestine, not so much in liver, but again binding in all of our tissues. No evidence for metabolic changes other than this reduction.

This study then demonstrated that the bioactivation of DMA to DMA(III), which binds to cellular thiols is evidence for bioactivation mechanism independent

of the methylation process and showed to us that risk assessments need to include both DMA and inorganic arsenic in risk assessments particularly they are both - the intake is of comparable magnitudes in people.

A study we just recently published is one on Rhesus monkeys where we looked in the same vein at the differences between adults and neonatal monkeys of different ages, starting at PND5 and then sampling the same individual monkeys at different ages and looking at the pharmacokinetics of blood since we now understand the information from erythrocytes and plasma give us really good complementary information to the mouse blood and tissue measurements.

The first thing we noticed was that there really were not - there were miniscule, insignificant differences between these young monkeys of different ages in both the kinetics and the evidence for metabolic activation.

The other thing we noticed was that the adult monkeys and the neonatal monkeys were actually quite similar in their processing of inorganic arsenic.

The next thing that we reported was that when you compare the adult monkeys with the adult mouse model after scaling for the different body weights allometrically, there really were not minimal differences between the adults of both species.

But what we did see, if you recall, is that given the strong evidence for metabolic deficiency in the neonatal mouse that of course meant that there were major differences between neonatal monkeys and neonatal mice in the metabolic activation and excretion of arsenic in metabolites.

And what this told us of importance is that toxicities identified in this mouse model may over predict those that you see in primates based on the significantly lower internal exposures, which is one element of toxicity. To rephrase that, the mouse model appears to be a sensitive model for exposures in primates based on internal exposure data.

The last paper that we are working on is one where we are looking at the chemical kinetics. This is not even in a cell, certainly not in an animal, but looking at the reactions of cellular model reactions of cellular thiols with arsenic species, inorganic and organic as well to understand how those chemical reactions will also participate in the observed metabolism and excretion effects we see in a whole animal.

We looked at kinetics and mechanisms for reactions between arsenite and glutathione and then also looked at the arsenic 5 analog arsenate and also the MMA(V) and DMA(V). And what these studies showed was that a few

things. First of all is that glutathione efficiently reduces these pentavalent arsenic species to the trivalent and even more is that ligand exchange between one set of thiol arsenic complex and other thiols is extremely rapid, which tells us then that there are roles for chemical reduction as well as enzymatic and methylation reactions in vivo. But that once these trivalent arsenic species are formed, they can shuttle between thiols as they work their way through the body and emphasize the role for the thiols, particularly glutathione, which is present at high millimolar concentrations in virtually all cell types and how these are important and in ligand exchange, in mobilization, and how they interact with molecular oxygen in the eventual urinary excretion in the overall disposition of arsenic in vivo and the associated toxicities.

What does all this mean to risk assessment? DMA is the common link in arsenic toxicology both for intake of inorganic and organic forms and that the methylation of inorganic arsenic and the reduction of DMA all produce significant amounts of tissue-bound trivalent DMA.

This goes to understanding the observations that both inorganic and DMA(V) are carcinogenic in rodent species. Unfortunately, this highlights a deficiency that many of the current risk assessments use inorganic arsenic

carcinogenesis alone. That is because there is very strong data from contaminated drinking water analysis in Taiwan that gives some level of dose response. Let us be clear. These highly contaminated drinking water sources that are used here. And, again, when you consider that rice and seafood both contain DMA and that the intake is comparable to that of inorganic arsenics is concerning.

And then finally, a new emerging element in the arsenic risk assessment that is starting really to come to fruition both from the chemistry side and now from the metabolism and toxicity side is that seafood also contains a couple of other classes of arsenic compounds, these so-called arsenolipids and arsenosugars, which are extensively converted again to DMA(V) in people and that these are major contributors to dietary intake. These have been studied very well in Japan. There is some emerging information in the US. Again, really, it is because modern analytical methodology that CFSAN chemists have pioneered really have made it possible to quantify these new more recalcitrant species in the diet so that this information is becoming available and will need to be included into the risk assessment to make sure that all the arsenic species are being adequately considered.

Again, just to sum up. In my opinion, this set of studies has really provided a hell of a lot of really

useful information if I do say so myself. It was because of a systematic approach to control dosing studies that allowed us to identify the toxic processes involved, which start with these fluxes of reactive trivalent arsenic species and that end up with DMA(V) being excreted into the urine. Again, this is a different process from the other heavy metals. Arsenic is a unique compound that when we understand the chemistry and metabolism a little better, it allows us to focus in on the key elements.

But, again, this whole notion of first pass metabolism had been ignored and would never have come out without systematic studies like this. Again, the key was to be able to focus in on the levels of these reactive intermediates, which takes a little ingenuity to make the measurements in an appropriate way, but it allows you to see so much more of the picture.

Again, recognizing that the dimethylated species predominantly allows you to focus on the really important ones. There is a lot of speculation in the literature of maybe these intermediates or maybe the substrate arsenite are going to be the more important. That may be true, but the simplest explanation is generally the correct one. You have the highest concentrations of a highly reactive species DMA(III). It is most likely that that is the most

important element, certainly one that needs to be dealt with first.

Again, the ability to use erythrocytes. This opens up the possibility to do human studies and get complementary information from kids and adults. You can start to understand the relative concentrations in target tissues and again brain, fetus and other tissues have much lower (indiscernible). It does not go away, but you have to assume some additional level of sensitivity, which is not always incorrect. Again, start where it is highest that are target tissues and work your way down to the lowest exposure tissues.

Again, I cannot emphasize the importance of dose non-linearity. It means the farther away in dose you get from what humans are exposed to, the less relevant it is. I just cannot say it any simpler. These high-dose studies probably are not worth a damn.

Again, we identified a whole new pathway for toxicity here and that is this reductive pathway that funnels everything into the same reactive intermediates. And the lactation transfer identified a key deficiency in the only animal model for arsenic carcinogenesis, which also highlighted the deficiency in neonatal mice if they are dosed adequately. They are going to have higher levels of these toxic metabolites than you would see in adults.

The other thing is - important elements for risk assessments or inter-species extrapolations particularly towards humans. That was why the neonatal monkey was directly compared with the neonatal mouse to understand if there are any extrapolations that we can make better by differential species measurements.

Again, I focus on the - where all of this has led my thinking is that we have laid the foundation to provide a key element needed in risk assessment, which is a dose responsive animal model that incorporates not only adult exposures, not only neonatal exposures where we have demonstrated additional susceptibility, but also the fetal exposures to really do an adequate measurement of carcinogenicity for both inorganic and organic arsenic forms all of which are needed in my mind to do the risk assessment of arsenic species in foods.

That is probably the last publication, the one I just - the chemical reaction is the last ones I will show. That is it for me, folks. But I would be happy to take any questions.

(Applause)

Agenda Item: Discussion

DR. DA COSTA: Thank you, Dan. It is really good work that you have done. It is not flashy work that produces --

DR. DOERGE: What are you talking about?

DR. DA COSTA: You have done everything in a very systematic fashion, which is something that IAA's(?) has enabled throughout the years and which I think is really important.

DR. DOERGE: We did the same thing with BPA and I told John Buker(phonetic) to his credit at the time that this kind of work does not get done without the full support of the NTP. I could not have been any clearer in my gratitude.

DR. FITZPATRICK: It is flashier to us -

DR. DA COSTA: We have time a few questions.

DR. FITZPATRICK: I said it is a headline to us if we have to consider DMA as toxic. Since it is in seafood, we have in the past not really considered DMA to be toxic. It looks like you are changing my mind.

I was interested in - were you able to tell in the mouse, where in the brain - because I read they think maybe it goes to hippocampus. That might be - but you cannot tell - I know, Sherry, you found it in the brain too, you found that inorganic, and you did find it in the fetus too. I know arsenic crosses the placenta. I am assuming DMA crosses the placenta.

DR. DOERGE: No, that was the finding that maternal metabolism reduced the exposure of the fetus to

all but DMA species. The inorganic arsenic was all methylated. Only the DMA was observed in the fetus.

DR. FITZPATRICK: Very nice work.

DR. HEFLICH: Some of this may be rather naïve, but it just struck me from what I remember about arsenic and genotoxicity that some of these metabolites are a lot more genotoxic than others and some of them have innate toxicity. Is it clear - assuming cancer is the endpoint of concern here, is it clear where the cancers come from? Are they due to - some de novo or genotoxicity or they are selective effect on background cancer drug - has anyone ever looked at this in either the mouse model or the human tumors?

DR. DOERGE: I am not aware of it, Bob. Again, these -

DR. HEFLICH: A simple thing that you could potentially do to sort this out where the actual effect is coming from.

DR. DOERGE: As you might recall, that is one of the elements that future studies would include are looking at these particular mutations.

DR. HEFLICH: The mouse model is good, but you sort of backflip around to get any kind of response. The question is how relevant that is to what is occurring in humans in my mind anyway. You could actually do that by

comparing the carcinogenicity based on what is causing the cancers, the mutations.

DR. DOERGE: The problem, Bob, is that it really - these trivalent arsenic species do not react with DNA. It is all indirect.

DR. HEFLICH: Some of these things are clastogenic. I can tell you that.

DR. DOERGE: Again, there are a lot of protein targets that are important in the regulation of faithful replication. It is indirect.

DR. HEFLICH: Is it their class to genicity that is important to their carcinogenicity or is it their toxicity that has a selective effect on preexisting mutations that result in the cancers?

DR. DOERGE: I cannot answer that, but I suspect virtually all that work has been done at pretty high doses. I would be very suspect to the relevance to the risk assessment.

DR. HEFLICH: You have lots of human tumors out there. People should be able to figure this out.

PARTICIPANT: You mentioned that based on the human studies and the margin of exposure is already close to one. If we add those DMAs and we add those sugars from seafood, what would be the --

DR. DOERGE: It will still be close to one.

PARTICIPANT: Because the inorganic arsenic(III) is still dominant in the food.

DR. DOERGE: No, they are actually quite similar. When I think all the seafood species are added up - again, it varies. Rice has different levels of inorganic and organic arsenic, but you add in - certainly when you add in the seafood, the organic arsenic is going to go up. In some situations, that will become the dominant form of intake particularly in developed societies where drinking water contamination has been greatly reduced. The problem is still the same. The margin of exposure is still way too close.

DR. BELAND: But Dan, isn't the point that that margin of exposure is based upon a very high exposure scenario. And what you are saying is if we drop the dose down to levels that are more realistic for let us say exposures in the United States, the margin of exposure would actually increase.

DR. DOERGE: Fred, that is exactly correct. It is even more interesting than that because there is a group that publishes out of Johns Hopkins that Stephen Lamb that has looked at the US arsenic carcinogenicity data. Their interpretation of these data is that only - that all of the cancer risk is being driven by wells with levels of arsenic above 50 or 100 that basically is skewed at the high end.

When you take those out, you actually see an inverse. Arsenic becomes protective at the low dose so it is even worse.

That is the problem with relying on heavily contaminated drinking water studies to use in a risk assessment because they are skewed by these levels of exposure that are a hundred or a thousand times higher than - that is why my - what I said when I saw this margin of exposure of one thing is that either we have a really serious problem or we are really doing something wrong in the risk assessment. I suspect it is the latter, but I do not know. That is just conjecture.

DR. FITZPATRICK: We did not take the organic species in account when we did the risk assessment. I think the Epi studies really just looked at high water concentrations. They did not really indirectly not by looking at what were tube wells(?) and stuff. But they did not take into account dietary exposure either. And seafood is the highest level of total arsenic, higher than rice.

DR. DOERGE: Susie, there are control groups - they estimated intake from drinking water was like 10 microgram per kilogram body weight per day. It is way above. That was the least of their problems.

DR. FITZPATRICK: I agree. Because here most people are not exposed to them - really only about 2 ppb in --

DR. DOERGE: Not to mention all the other elements of using developing world populations to model nutritional sufficiency and all kinds of things, educational enrichment. It is tough.

DR. SLIKKER: Really nice presentation. Thank you for this great overview. I was thinking about this in terms of all the variables that you brought up, including the whole life exposure, the exposure during lactation, the difference between metabolism, especially based on not only developmental stage, but on species. It seems like it is really ripe for modeling. I was wondering if you were thinking that bringing some of the data together now in a modeling type exercise that allow you then to be more capable of moving between species and determining where the main emphasis should be.

DR. DOERGE: Data sets like this definitely have an eye on modeling when they are designed and implemented. Those elements are not lost. Like everything else, it is a matter of resources.

DR. FITZPATRICK: What would you think if you were asked what the next steps should be then? To do modeling?

DR. DOERGE: For me, modeling would not take you any further than what this has done to identify issues and margins and things. To me, what is really needed is an adequate dose response analysis. That is my own opinion.

DR. HUNT: Just a quick question. Maybe you answered this in a previous meeting that I missed. Why didn't this study include developmental milestone acquisition, some more general toxicity parameters? Was this covered before?

DR. DOERGE: These were kinetic studies. They were not toxicity studies. Those were meant to be addressed and follow-on issues that have not started yet.

DR. HUNT: In terms of the FDA following the three R's, is that something that we should continue to do? In other words, we have a batch of animals that we are going to do this type of test with, if one group looks at toxicokinetic parameters, why not have another group also be doing those other tests so that we are reducing the number of animals that we are using?

DR. DOERGE: We have done a few studies like this and particularly those studies, toxicity studies that are used for regulatory risk assessment, the requirements for the sanctity of those animals is such that you could take off excess pups and things and do the kinds of measurements that we have done here, but to develop, do all the repeat

dosing and the extensive dosing. These really are standalone studies that precede subsequent bioassays because again as we shared here, a lot of elements in metabolism that directly impact the way you conduct these bioassays. These really need to go before the bioassay.

Clearly, designs that we have done in the past have used things like excess pups to make measurements in the same animals from the toxicity studies so that we have a direct link between the pharmacokinetic measurements and the toxicity testing. Those are important elements that we try to address.

DR. DA COSTA: Thank you, everyone. There is probably more discussion to still be around this subject area. Thank you, Dan.

Dr. Ferguson is going to be presenting the next talk, giving an update on the behavioral side of the arsenic studies.

Agenda Item: Arsenic Behavior Study, Update

DR. FERGUSON: Good morning. Most of you are aware that we are in the middle of a study of the developmental neurotoxicity of inorganic arsenic exposure. What I am going to provide to you this morning is just an update of where we are in the study.

Now, I will show some results and I will show some data, but please consider these data to be preliminary

in nature until the study is finished. And the two critical people involved in the study, Tim Flanigan and Andrew Shen, work much harder on the study than I do. And Andrew is here with me today.

Dan really set the stage for the necessity of studying arsenic exposure. I will just briefly review the study design that we are using. Arsenite or arsenic(III) is our study compound. And we are using Sprague-Dawley rats.

Now, Dan's work and the work of others have shown that most commercial rodent chows have measurable levels of arsenic in them. We are using a purified chow that contains very low levels of arsenic. We set some of this chow to Charles River, our rat supplier, and they put the animals on this chow the morning after breeding and they are shipped to NCTR. They arrive on gestation Day 5 and they are maintained on that low arsenic chow.

The pregnant dams are gavaged twice daily, starting on GD6 until the day prior to parturition. And the offspring once they are born receive on daily gavage on postnatal Days 1 through 21.

These are our doses, vehicle control, which is in 18 megohm deionized water and the arsenic doses are .1, 1.5, 3.75 milligram per kilogram per day. Those doses are divided in half for the doses in the pregnant dams such that the two daily doses sum up to that total daily dose.

Now, you might remember in our preliminary study where we dose the animals in their drinking water, using arsenate, we found decreased gestational water intake in the pregnant dams. And the graph on the right shows you there are three arsenic treated groups, low, medium, and high group. It was almost a dose response with - and this is shown with respect to controlled water intake. During gestation, our pregnant dams drank much less water than we anticipated they would, much less water with respect to the control group. That is the reason for going to gavage in our pregnant dams in the current study.

Arsenic is supposed to be odorless. It is supposed to tasteless. It is the poison of king. It is the king of poisons. We do not have any explanation for this decreased water intake during gestation. We know that it popped right back up to control levels when the animals are put on control water after parturition. But there is at least one previous study in the literature and I believe that one of Dan's studies also found decreased gestational water intake.

We do have data from the current study, however. This is gestation water intake, our current study. These are complete data. And in this and all of our subsequent graphs, the legend will always stay the same. It will always be the control animals in the black. Throughout

gestation, there were no arsenic effects on water intake. And water intake is measured daily.

Lactational water intake as well was unaffected by arsenic exposure. Food intake. Food intake is measured daily throughout gestation and lactation and there were no significant effects of arsenic on food intake nor on lactational food intake.

I think the next graph shows gestational body weight. Throughout gestation, of course the dams gain weight and there were no effects of arsenic exposure. This is actually good news for us because the focus for us is on cognitive effects in the offspring, the effects of arsenic on the developing brain. Those can be confounded if we have decreases in gestational body weight, decreases in food or water intake in the dams, which might affect maternal behavior. And lactational body weight, also unaffected by arsenic exposure.

On postnatal day 0, the number of pups per litter are counted and sexed. Shown here are the percent of males per litter. Each circle represents a separate litter. Sex ratio was not affected by arsenic exposure.

Now we have some very good chemists at NCTR and these chemists assayed our gavaged solutions. The graph shows you - the X-axis shows you the targeted concentration of those solutions: .1, 1.5, and 3.75. And the X-axis shows

you the measured concentration. You can see with the percentages there that our targeted or gavaged solutions were right on target. The two data points that are shown there that do not have a percentage where sample or practice solutions made by our chemists and they were also on target.

Now we also collected brains from the culled pups. At postnatal day 1, pups are culled to eight pups per liter, four males, and four females. We have a number of excess pups. We took the brains from those excess pups and stored them in our minus 80 freezers. And any pups that were not retained past weaning, we also took those brains and stored them. Those will be assayed by Mirek Styblo at UNC.

In the preliminary study that we did a few years back, we did send some brains to Dr. Styblo's lab at UNC and there are measurable levels of arsenic in culled pup brains. As Susie mentioned, it does cross the placenta barrier.

We have very few pre-weaning deaths. These occur in any study. Eight pups died prior to weaning. They are scattered throughout all four of our dosing groups and across both males and females.

Now this study was designed with seven cohorts of dams, each spaced a month apart. Each cohort was 16 to 20 dams and we had no dam deaths.

The pups in each litter are weighed daily. Shown here on the left are the males and on the right re the females. All of our arsenic-treated groups gained weight just as the control pups did.

For those pups that we do not retain past weaning, we do take the whole brain out and weigh it. The left shows the male whole brain weight and the female whole brain weight is shown on the right. This is postnatal day 21. Male brains are significantly heavier than female brains at weaning. But there were no arsenic effects.

Now, we have a number of neurobehavioral endpoints that we are collecting. It is quite comprehensive. The live animal portion of this study will be finished in January. Several sensory motor assays. These are particularly important for any assessment of cognitive abilities. If an animal shows what looks to be a learning deficit, we have to be sure. As scientists, we have to be sure that that particular learning deficit is not a result of problems with motor capabilities, problems with sensory capabilities. To assess those, we use rotarod, which is a measure of motor coordination, grip strength, acoustic startle and prepulse inhibition and in-cage wheel running.

And then our focus here is on the neurocognitive effects. These are assayed by Morris water maze performance, 8-arm radial maze, Y-maze, open field, and the light/dark test. And I will show a couple of examples of these assessments.

The first one I want to show is home-cage behavior monitoring. There are some scientists in the field of neurotoxicology that feel that we should be looking at more ethologically relevant behaviors in our rodents. That is, rather than taking the animal out of its home cage and placing it into a test apparatus, we should be looking at what the animal expressed naturally. For lab rodents, the only thing that they can really express naturally is what they do in their home cage.

This is what the setup looks like to the rat. They are in their normal home cage. You can see the sipper tube or the water bottle. You can see the bedding on the bottom. They are placed in a room that is in the same light-dark cycle as their home room. This is a normal cage rack that we set cages on. Humidity and temperature are monitored just like in their home room. Everything for the animal is identical to their home room.

Now, each home cage has a camera facing it. Shown here on the left is a video stream that the camera has picked up of an animal behaving. You will see the animal is

rearing. Typical lab rodent behavior. That video stream is fed into the computer and the software will generate behaviors for us. What you will see on the right is that same video stream. You will see letters by the animal's body parts. And those letters represent a specific body part.

From this, the software gives us frequency and duration of behaviors such as walking, rearing, and rearing is even differentiated into supported rearing by placing the four paws on the cage or unsupported rearing. We get measures of inactivity, eating, drinking, and we get these by - is it a one-hour period? We actually delegate the period that we want it by. We can look at Circadian rhythms in these animals. We can look at when we place them into this new cage, the habituation to the new cage. We think that this is going to be a very sensitive measure. The animals are in here for three weeks, three consecutive week period. It gives them time to adapt to that. This is something that we have used in the past, but we have not used it as long as three weeks. We used it for 24-, 48-hour periods.

Morris Water Maze. If you are familiar with any sort of behavioral assessment, Morris Water Maze is sort of the gold standard for looking at learning and memory problems. The left shows an overhead view. It is just

basically a cylindrical tank filled with water. The water is made opaque by the addition of nontoxic paint. The animal cannot see through the water. And slightly below the level of the water surface is small platform. And you can vaguely - I do not know if you can see it from your side here. It is below the level of the surface of the water.

The animal must use extra maze cues outside the maze, these are on the walls of the room, in order to locate the platform. You can see on the right is kind of the rat's eye view of the tank.

Animals are placed into the tank four consecutive days, three trials per day. Generally, they have no clue that there is a platform in there. They have no way to find the platform. They typically do not find it on the first few trials. If they do not find the platform within that two-minute maximum trial period, they are gently guided to the platform by the human tester that is in the room. They are allowed 20 seconds on the platform. And usually what rats will do when they are on the platform is they typically rear up and they look around the room.

What we think they are doing, we do not know of course, but what we think they are doing is they are basically orienting themselves to where that platform is with respect to the pool so that the next time that they are placed into the tank, they know where the platform is.

Their behavior is automatically measured. There is camera directly above the pool and tracks their behavior. Typically, this is done - you saw the black tank and the white rat. It is done by the contrast between the black and the white. The software allows us to design what we want within that tank. You can see that we have designed four quadrants and this is typical for Morris Water Maze. The red dot there shows where the platform is.

We also have a measure of what is called thigmotactic behavior. Rats do this quite a bit. It is swimming close to the walls of the tank or staying close to the walls of an apparatus. You will see the tracking for day 1 trial 1. This is an animal that has never been in the pool before. And from the software, we will get swim path length. That is the distance that the animal swam. We will get the latency to locate the platform. And we will also get swim speed. Swim speed is a really important measure in Morris Water Maze performance. A number of labs do - this guy did not find the platform and he will not find the platform. A number of labs will measure Morris Water Maze performance using a stopwatch. You place the animal in the tank, start you stopwatch, measure it until the animal finds the platform. If that is all you have, that is fine.

But because drugs and toxicants can affect motoric capabilities, it is really important to measure

swim speed. If you use a longer latency due to a drug, that longer latency may be because the animal is swimming slower, not necessarily because of a learning deficit.

The graph on the right shows that same animal on its very last trial. It is headed directly for the platform, reorients itself and locates the platform. Now, the animal cannot use a strategy such as I am going to swim straight and then make a sharp right. That strategy will not work because the animal is not always placed in the tank on that side. It can be placed on the tank on any number of locations. It definitely has to use those extra maze cues to locate the platform.

We know that rats are - they can cheat. Rats can use the urine trail of the animal that has just been run before it to locate the platform. We know that. To prevent that, we use a small paddle and gently stir the tank in between trials. They are cheaters.

This is done for four consecutive days. On the last day, we do a probe trial and that is typically done in Morris Water Maze assays. On that probe trial, the platform is removed and the animal is given only 30 seconds in the tank. And if an animal knows where the platform used to be, it will spend a lot of time swimming in that particular quadrant. If the animal does not remember or is unsure of where the platform is, it will swim more often in the other

three quadrants. The probe trial is a measure of memory. The first four days are a measure of learning.

We are collecting brains for neurochemistry and neuropathology. Those assays are scheduled to begin in February. They will consist of histopathology of the culled pups. That is postnatal day 1 pup brains. Those pups that we did not retain past weaning, PD21. And then the adult offspring brains - our live animal studies end at postnatal day 180.

We will also do western blots on the brain homogenate and isolated brain endothelial cells. And we have scheduled multiplex assays and ELISAs on serum. Those assays will look at hormones, cytokines, and growth factors.

Now unrelated to our NTP agreement, we are looking at a couple of additional endpoints. With Johns Hopkins University, we completed a material transfer agreement with Phena Silia's(phonetic) lab and Phena is interested in the effects of environmental exposures during pregnancy on later offspring lung function. We have sent her lab lung tissue culture and serum. We sent those samples blinded with respect to treatment. We will not unblind her lab until our manuscripts are published.

With the division of systems biology at NCTR, we have an addendum under review now. This is for Ellen Jones

to do MALDI imaging and proteomics and lipidomics, which will target oxidative stress pathways.

Finally, I would like to acknowledge Suzy, who is not in the room. Suzy Fitzpatrick of CFSAN, who has been an incredible help with experimental design and interpretation. Delbert Law is the hands-on person in our lab. Dr. Elvis Cuevas-Martinez and Hector Rosas-Hernandez are our neuropathologists and neurochemists and they are assisted by Bonnie Robinson and Susan Lantz. Thank you.

(Applause)

Agenda Item: Discussion

DR. DA COSTA: Thank you, Sherry. I have one question. I was curious. I noticed that on the cage where the rats were monitoring by the video system that you do not have environmental enrichment devices. Wouldn't they create more of an opportunity to ascertain more complex behavior rather than just - or does it complicate the video analysis?

DR. FERGUSON: It complicates the video analysis. Our software cannot tell the difference between a stationary rat and a stationary environmental enrichment tube.

DR. WALKER: What was the system that you were using?

DR. FERGUSON: For the home cage monitoring, it is by CleverSys. They actually have a number of systems out. They have systems out to monitor non-human primate behavior. They have systems out to measure social behavior between two rats.

DR. BELAND: When you are having these measurements made of the arsenic levels done at UNC, are they going to also look at other tissues to see if there is differential like Dan described where the brain is much less than say the liver and so forth?

DR. FERGUSON: I wanted to come back to Piper's comment earlier and that is that we save a lot of different tissue samples. My lab is not necessarily interested in liver. My lab is not necessarily interested in kidney, but we have saved those samples in our freezers. It is possible with - our funding right now from NTP does not allow us to send additional samples to Dr. Styblo's lab, but we could because we have those samples.

DR. FLANNERY: I just have a quick question. Brenna Flannery. Thank you for your elegant explanation, Sherry. I have a couple of questions for you regarding the behavioral assays. You indicated that sometimes the animals will exhibit the thigmotaxis behavior within the Morris Water Maze. Is one able to draw any conclusions about anxiety-like behavior from the amount of time that they do

that? I am wondering then if we could possibly get some of that information from that test.

DR. FERGUSON: I know you have an additional question, but I can only think of one question at a time. We also are doing open field behavior and we measure thigmotactic behavior in that. We are also doing elevated plus maze. No, we are doing light-dark as more direct measures of anxiety.

Typically what animals do in the water maze is they do a lot of thigmotactic behavior initially on the first few trials because they are really scratching at the walls, trying to find an escape. But that goes away. We have more direct measures of anxiety with the light-dark test and open field behavior just typically by the sides and also entries into the center.

DR. FLANNERY: Thank you. I am just also curious about the home cage behavior monitoring. Initially when you start that, do you put them in a fresh cage?

DR. FERGUSON: Yes. Absolutely.

DR. FLANNERY: And then they stay, but I know that that can also affect behavior. Do they stay in that fresh cage then for three weeks?

DR. FERGUSON: No, their cages are changed once weekly. They are in there for three weeks. Twice a day our animal care personnel come in for two minutes at most,

maybe five minutes at the max and do what is called a death check, make sure the animal still has food and water, everybody is still alive, cameras are still running. And then once a week, we go in and it takes maybe 20, 30 minutes to change cages and we do that during the day when they are less active. Yes, they get a new cage once a week.

DR. FLANNERY: I am just wondering then if that can sort of go into what Goncalo was saying in terms of it is going to be novel. While there is not an object in there, perhaps it will be interesting to see if the behavior is different in terms of the first day when you put it in the new cage versus after it has time to get used to --

DR. FERGUSON: We know this not only from home cage monitoring, but just from our own observations. Every time animals have a cage change, there is increased activity even though it is during their sleeping time. There is increased activity. There is sniffing. There is a lot more activity. That is going to show up in the data as well.

DR. FLANNERY: It would be interesting to see if arsenic affects that at all. Thank you.

DR. DA COSTA: Seeing that there are no more questions, I think we are going to break. We will meet again at 10:45. Thank you.

(Break)

DR. DA COSTA: Let's reconvene. Our next presenter is Dr. Khare who is going to be providing an update on the work that she has been leading on the microbiome. This work has been going on for a few years now. The main objective of the talk will be to provide an assessment of what we have learned, discuss a little bit where we should be adding. This is definitely a very complicated field, so it is going to be an interesting discussion.

Agenda Item: Microbiome, Update and Next Steps

DR. KHARE: Thank you. I am here to give you an update on the assessment of the role that the microbiome may play in the toxicity of the xenobiotics. And this is an interesting interaction between NTP and NCTR for the capability building for the microbiome assessment on the toxicology studies.

So today, we will start with the goals of the microbiome program, go over the capabilities and what each tool has told us. Then I will give highlights of the results for the xenobiotic assessed to date and then what the capabilities have told us. Then from animal to human relevant translational model and where we are. Then the path forward for the future assessments in the phased approaches and then the questions.

So the goal and objective of this program is to establish standardized approaches for the sample collection, methodology and intestinal microbiota study. And second, for the data analysis, data repository and data presentation.

And second, to use these approaches to conduct host microbiome assessment of the NTP/NCTR studies to determine if during the xenobiotic exposure, microbiome is adversely impacted. And assess if various chemicals through multiple modes and routes of exposure have an impact on the microbiome. And also to develop the approaches for the translational research to determine the host microbe and xenobiotic interaction. These efforts are done to move us forward, to modernize the risk assessment of the xenobiotics.

So as far as the research capabilities, the division of microbiology is equipped with several research tools to answer for the xenobiotic microbe interaction, as well as xenobiotic host interaction. And these research tools are in vitro bacterial culture. Then also the ex-vivo system and the animal models.

And for the host interaction, we have in vitro cell culture system, and whether it is single or the multiple cell type or the organoid. And then we have ex vivo system and then the animal model. So with the

xenobiotic microbe interaction, we define the life bacteriology, basically who is there. And with the omics approach, we define what they are.

And for the animal model, we are looking for the whole biome approach, where we are defining the host, microbe and xenobiotic interaction. Now, these research capabilities have been used to address the impact of several compounds. And those are mentioned here. I will be giving the highlights of the results, like selective results, for some of these compounds.

The results of these, you will see that they are a step towards regulatory agencies readiness to evaluate innovative emerging technologies for improving the risk assessment of the xenobiotics. First, I will start with arsenic.

Here, we have done the risk assessment of arsenic, looking for the dose and the route of exposure. And the goal for this study was to assist the potential toxicity of the short-term arsenic exposure using oral or intravenous route. And here, I would like to emphasize, and there was a question also considering the three Rs. These are the animals those are used for these studies. We took the gastrointestinal tissue and fecal sample to assess these host microbiome interaction.

So our first was to see whether that the intestinal microbiota diversity and host associated responses in terms of immune mediator and integrating has been changed. And second was to determine if the outcome of arsenic toxicity depends on the route of the exposure and if it has any relationship with the arsenic speciation.

Why we are studying this microbiome and the arsenic because we know that the arsenic transformation with the toxic form is likely carried out by intestinal bacteria. And it may alter the intestinal homeostasis.

Some of these results have been already published. I will provide results or the highlights of the results here for the oral in which three different doses were used, and compare it with the intravenous.

And the point that we noticed here, the results on the single. And in oral studies, samples were collected 24 hours or 48 hours later. And one part of the result is also that eight days repeated exposure. And for the intravenous, it was just the single exposure. And samples were collected 1-hour, 4-hour, 24-hour and 48-hour after the exposure.

So first, we start our analysis with the live bacteriology. We have the animals. We collect the poop, and then come directly to the lab. And within three days, we have the results. And here as you can see the results,

low-dose, medium-dose and high-dose are represented towards the right side. And then here is the control exposure.

Then 24-hour, 48-hour and repeated dose, you can clearly see that there is the difference in the bacterial recovery during these exposures.

We went ahead and did the 16s analysis because with live bacteria, you can just do the selective bacteria and cultures. But with the 16s or omics approach, you can assess the global analysis of the bacterial species. And here, examples of some of the species are given. Those were impacted by the arsenic exposure.

So what we found that in the adult total aerobic population was increased. And the anaerobic population was decreased in the repeated or single exposure. Predominant bacteria that were found after the single exposure was associated with the disease, like bilophila. And then low butyrate producing bacteria were there.

But when we had that repeated exposure, and also the high-dose exposure, we found that there was bacteria. Those were producing low butyrate production. The bacteria population was decreased, therefore responsible low butyrate production.

And also there was an increase in the bacteria number. Those were associated with the detoxification of

the arsenic. All had the arsenic resistance gene and were also involved in the thiolation of the arsenic.

Next, we look for the intravenous exposure of what happened in the intravenous exposure. Here again, we see that within one hour, the lactobacillus population that is one of the prominent bacteria was decreased. Except as you see here, bacteroides that comes up at 24 hours. The rest bacteria had negative impact.

And we know from the literature that the bacteroides has the arsenic resistance gene. And we think that at the 4-hour, whatever bacteria is remaining there, maybe they have the arsenic resistant gene. And they will come up in 24 hours. Then it will be the survival of the fittest at the 48 hours.

Then again here, we look for the species abundance. And here are the predictive rules of the species are given. And here we have done the fecal, as well as ileum associated bacteria.

And what we see here, whether we see ileum or the fecal, and I want to bring your attention because the population is not always the same as found in feces and what is ileum-associated bacteria. But here if you see this, what we found that the bacteria, those that are present here, are either like they are involved in the disruption of the first protective layer, whether it is

fecal or ileum. Then the epithelial layer disruption it is causing, then there is pathogenic bacteria increasing, and it is suppressing the immune responses.

Then we went ahead and did the host responses also. And first, I would like to bring your attention to the oral exposure. And we have done the cell-cell junction. Here are two cells. And this is the IP analysis after the gene expression has been given.

When I show the next result, you will see that the cell-cell junctions were not altered during the oral exposure. Whereas the significant perturbation of the immune response related genes were up during the oral exposure.

But when we look for the IV exposure, there was down regulation of the cell-cell junction related gene. And each of these green dots is actually one gene and thus has been down-regulated. Whereas for the immune perturbation, after initial, there was no impact on the immune responses until 48 hours.

For this then, we came up with this mechanistic diagram. And I used the results that were obtained from that speciation study by Dan Doerge also. And in this, if we look for the oral gavage, we see that low intestinal or there were no changes in the permeability, but there was high immunotoxicity. Whereas when intravenous indication

was given, there was high intestinal permeability. And then there was no changes in the immunotoxicity.

And what we predict here that this is due to maybe the convulsion of arsenic into more toxic trivalent and pentavalent forms that was found during the oral exposure. Whereas in the IV exposure, these species were not found, and probably that is why there were not much changes in the immunotoxicity. But it may cause the permeability changes due to the direct exposure.

So next, I will give the results for the triclosan study. This was done to assist the developmental toxicity of the triclosan for the microbial diversity and abundance. Correlation between the microbial diversity had IgA in feces, and the gender bias after 28 days of exposure.

And why the microbiome study, we wanted to do it in the triclosan case because, and this is known as an antimicrobial agent. And there are conflicting reports about the antibiotic resistance and gender effects.

In this study, this was done in collaboration with the Dr. Sutherland at GD6, these animals were orally gavaged with 100, 500 or 1000 milligrams body weight. Some animals were sacrificed at GD18. The exposure continued. And then the pups were sacrificed at PND7 and PND28. The

PND28, the dams were also sacrificed. And pups were given the oral gavage from PND12.

Here, we have done the 16s sequencing, TCS detection and IgA analysis apart from the other usual parameters. But I am going to give the results of these parameters today.

So by doing the 16S sequencing, we can see a high abundance of proteobacteria. Those involved generally the pathogenic bacteria. Whether we see at GD18, PND7, it is not that obvious but by PND28, you can clearly see that there are differences and significant differences into the mean as compared to the control. And also in the dam, there were significant differences.

Then we looked for the genus analysis. This gives us a very unique pattern. Here you can see that all the controls and all the respective groups are in the middle. And the abundance of this, if it is more towards the peachest color or reddish color, it is more abundance. And the blueish color is less abundance of these bacteria genus. And you can clearly see a dose-dependent gradual shift in the abundance of bacterial population here as you can see for the 100 or the 500 milligram for 48.

Now, we wanted to see whether the bacteria itself, how it correlates with the OTU. And you can see here that for the dam, there was always whenever the TCS

exposure was there, the number of bacteria increased in dam. And at PND28 in dam, the male pups. But not in the female. Rather in female pups, it shows that it is the sequence or the OUT is decreased.

Then we know that IgA plays a major role for the microbial diversity. So we looked for that IgA label. And again, we can see a correlation between the dam and male pup. But there was no correlation in the female.

So here, we can see the secretion of higher amount of IgA was more evident during the early development, as well as during PND28 and the dam. I am not showing the results of the TCS, but TCS was also present in the fecal sample in these animals.

The next study was gestational and lactational and postnatal toxicity of the BPAF. And the goal of this study was to look for the toxicity of BPAF exposure via feed on the gut microbiota and the immune response. At the early developmental stages, that is PND4, and those changes that occurred at the early stage sustain for PND 28 and week 13.

The BPAF is more studied because it lives in the environment more time as compared to BPAF. For this, again the rats were exposed from GD 6 real feed at 0 to 150 or 1,000 ppm. And the animals were sacrificed at PND 4, PND 28 and week 13.

So here, we did the first analysis of the microbiome by looking at the primer-based(?) analysis. Unfortunately, we were not able to collect the fecal sample for the live bacteria cultures, so there is another preliminary approach that we can do the targeted analysis. At the phyla level, we were not able to see any changes. But at the genus level, you can see here, there were significant changes. Then we did the 16S sequencing. And again, there were differences in PND4, PND28 and week 13 during the exposure of the BPAF.

So for the BPAF analysis, we went further ahead. So until now, whatever it is that I have presented, there will not be any based analysis. They just say what is there. But they do not say what they are doing. So we looked for that metatranscriptome analysis. I am giving the results of these and for all others on the way.

And what we see here, I want to give you one example. Here, out of all of these, in PND28, we can see this orange bar. And the orange bar actually goes for the degradation.

So using this metatranscriptomic approach, we can also say, okay, what is bacteria doing into the gastro intestinal tract. Until now, whatever I have presented, these were using the animal model. But we also the individual approaches versus the intestinal toxicity. And

we have at NCTR studies that have shown that where either via aloe vera leaf extra or by the purified aloin, there was known toxicity in the intestine. And it was evidenced by the goblet cell hyperplasia and the incidence of the adenocarcinoma. But the knowledge gap is what would be the molecular mechanism. From our lab, it is basically in terms of the microbiome.

For this, we looked at individual culture. We took the pure bacteria and incubated that with aloin with different concentration to look for the minimum inhibitory concentration. And what we found is that aloin lacks the antimicrobial property of some of the bacteria that is lactobacillus, Bifidobacterium and eubacteria.

And aloin was able to kill the lactobacillus in the aerobic environment. And then it was present in ileum, not in the colon. So basically, in the colon, it is the anaerobic environment, lack of oxygen. But in the presence of oxygen that is shown in the ileum, aloin was able to kill the lactobacillus.

And aloin also exhibited the antimicrobial property against enterococcus and akkermansia. These two are really important because enterococcus plays an important role. And akkermansia is mucin-degrading bacteria. So if more mucin is there, it will help to

degrade. And that may be the cause of the goblet cell hyperplasia in the gastrointestinal tract.

And we also did the aloin metabolism by intestinal bacteria. We show an example here for the eubacteirum and enterobacterium faecium. And it clearly shows that aloin is converted into aloe-emodin by these bacteria.

So apart from looking at the pure bacterial culture, we also took the rat fecal sample and incubated it with the aloin. And then looked for the butyrate production. We can see here by 24 hours, there is a decrease in the butyrate production. This was further evidenced by the lower abundance of the bacteria. Those are producing the butyrate.

So in summary, what we have shown here is that in vitro bacterial culture, aloin can decrease the live bacterial population. And then also the low abundance of butyrate production may lead to the decreased butyrate production. And whole cell result that I haven't shown here, but we have shown by the in vitro cell culture that the aloin was causing the disruption into the intestinal barrier integrity. And it also increased the cell proliferation and inhibited the proinflammatory cytokines. This could result into the intestinal hyperplasia and adenocarcinoma.

And we have been doing the silver nanoparticle study in collaboration with Dr. Mary Butral (ph.). And for this, our goal was to address how the ingested nanoparticle may influence the intestinal microbiome. Then we have also done some crosstalk between microbiome and host, epithelial permeability, gut-associated response and development of resistance to silver and other antimicrobials.

Some of the results, I will be presenting here. And in this study, we have used all three approaches, in vivo studies using the animal model, in vitro studies and also related it with ex-vivo expanse.

Here, we show that mucosa-associated bacterial culture, this is the study in which that is both male and female was gavage for 13 weeks, twice daily with three different sizes and three different doses of each size of the bacteria. But what it shows here is that bacteria property was size and was dependent. The smaller the size and lower the dose, it was more detrimental for the bacteria.

We took those samples and did the 16S analysis. What we found was there was male and female differences for the abundance of bacteria, as well as some of the host responses. So akkermansia, that is the mucin degrading bacteria, was increased. And then there was intestinal epithelial permeability that is maintained by allobaculum

and parabacteroides that also helps with the immune status that was differentially expressed or differentially present in male or female. And this corelated also of the studies with the gastrointestinal homeostasis.

So now from this, we wanted to do from animal to human translational research. And this was done by using the in vitro cell cultures and also using the ex vivo. Those are obtained from the collaborative human tissue from which the human samples are sent to us.

And we make the biopsy punches, incubated with the compound of interest. And we have shown that they maintain their integrity, and some of the results have also shown that they have the dose and size dependent responses for the silver nanoparticle exposure.

As you can see here clearly that the abundance of some of these bacteria levels changed due to the silver nanoparticle exposure. It correlated with the animal studies too.

Another study, we call it a proficiency study, is also under way in which we are evaluating the animal model, vehicle and the sampling size. So for this study, both rat and mice are incubated with the corn oil and water. And samples were collected from fecal, ileum and the ileum-associated bacteria, colon-associated bacteria and bacteria present in the fecal.

What we find is that there is a differential response by the rat and mice for the corn oil. And also those results are correlated with the intestinal permeability and the immune responses.

Now for the study outcome, like our overarching goal for this study was to predict the systematic toxicity where assessment of the intestinal toxicity in terms of impact on the microbiome and the gut mucosa-associated responses. The last study that I just defined, the proficiency study, we have identified distinct characteristics of the rodent model. We have used rat and mice. For the toxicity assessment. And then the corn oil was used a vehicle.

And it is really important because some of the xenobiotics that are not dissolved in the water, generally when the vehicle is used. So vehicle, per se, is having an impact on the gastrointestinal tract, it is very prudent to understand. That is what this study is.

And for the arsenic study, we have identified potential toxicity of single or repeated oral exposure and adult rodents. We have also provided results for the developmental toxicity of arsenic due to the single oral gavage. Those results, I haven't presented in this study. And we have shown that the route of exposure, whether it is

oral or intravenous, how it impacts the microbe population mediated host responses.

For BPAF study, we have shown that dose, duration and gender-based differences during gestational exposure via the feed. For triclosan, also we have run a developmental study. In this, the oral gavage dose was there. And we saw the results were sex dependent manner.

And in the aloin study, we have provided the in vitro model to assess the impact of the xenobiotic that can be used for the bacterial community, short chain fatty acid and the epithelial cell permeability.

The silver nanoparticle study, I know I went very fast through all of these studies, but each of these studies can be explained in 45 minutes. But for a silver nanoparticle study, the translation from animal model to human ex vivo intestinal explant system we have established in our lab. We can have male and female both explant, and how it is more relevant to the human. That is currently under way.

So these results have provided insight for the animal dose route frequency-dependent impact. Live culture analysis, as I mentioned earlier, that we can get the first quick readout within three days. It can also tell us whether further analysis of the microbial population b more high-throughput assays can be done.

Then one of the major advantages, what we found with the microbiome assessment, that we can see the effects at the lower doses which may allow for the determination of unaccounted toxicity. Then we have identified microbes from phyla to species level that may be contributory factors for initiating the perturbations in gut-homeostasis.

Metatranscriptomics study that I have shown for the BPAF identified that functional changes during the exposure of the xenobiotics. Then assessment of microbiome at early developmental stages, most of the studies were like arsenic, a single dose, or just the early developmental study.

But these studies further provide evidence whether we can predict the issue earlier on for the long-term study. And definitely, we need long-term studies after the single or the short-term exposure. And whether we can go into the phased approaches for doing the long-term studies.

And for the phased study, we can do several approaches. We can start what is in peach color with the live bacteriology, looking only at the microbiome, either by the live bacteria or the in vitro fecal culture. And the output or endpoint could be the metabolism, live bacteria analysis or who is there and what they are doing.

For the eukaryotic culture, we can use in vitro culture and then we can get RNA and protein. And then we can look for the intestinal gene expression for the endpoint for the whatever array or whatever path we want to assist. For ex vivo study, we can use including the fecal sample. We can also do all the peach color and the blue color. But when we have the animal, we can study the route, dose, frequency, developmental and all other kind of studies.

The future plan with this is to now perform a comprehensive analysis of microbiome studies that are ongoing to determine whether there are correlates for the immunotoxicity endpoint and the microbiota endpoints. And also to determine if there are correlates for the developmental toxicity endpoint and the microbiota endpoint.

And then a thorough analysis for the developing pup microbiome would help identify the tipping point for other developmental landmarks. This can be also done by fecal analysis just without sacrificing the animal also.

Meta-analysis of data will identify the core or the specific set of the gut microbes that could be associated with xenobiotic exposure. And finally, the ex vivo translational model could be used to identify gender-based differences in the more human relevant model.

And with all this, the idea for this research is can microbial dysbiosis be added as an endpoint for the hazard or preclinical safety assessment. And how all these studies contribute to the FDA strategic plan for advancing regulatory science and innovation.

So one of the strategy plans is to modernize the toxicology to enhance the product safety. And what we have presented here led us to realize or think about consideration of the additional endpoint in the safety assessment of xenobiotics which supplement the traditional metabolism, PK, PD, toxicity and tissue reside disposition information used in the traditional toxicity risk assessments.

And with this, I would like to thank our research team. Dr. Cerniglia has been really supportive of this study. We have Dr. Gokulan, Dr. Lahiana and Mr. Kumar in the lab who really went forward with this work.

We had collaboration with Dr. Dan Doerge and Michelle. They provided us the samples. This project started with Dr. Paul Howard and now Dr. Goncalo. Then our past lab members have also played contributory for this project.

Finally, collaborator, Vicki Sutherland and Nigel Walker. Their feedback has been really productive to bring these results on. Thank you.

Agenda Item: Discussion

DR. DA COSTA: I think it was a very well-organized by study description of all the work that we have been doing in the last few years. And where do we start here?

Clearly, it is probably one of the most complex datasets that we have had here in terms of trying to make sense of what are the commonalities, what is it where we should be aiming. If we were to start thinking about commonalities, do you see any line that is common, changes that are common among toxicants? I mean, of course, we are talking about very different toxicants. But is there any pattern that you are seeing that can inform where we should be adding?

DR. KHARE: In talks of the microbiome or microbiota, so we have some of the indicators, we know that some of the phyla, they are mostly for the pathogenic bacteria. If we see a high abundance of that phyla, we see, okay, there is a dysbiosis. So through that, we come from the phyla level. You can go to the species level. But like as you go to the species level, now there are thousands of the species.

It is not like, okay, phyla level. Okay, when we can't go to phyla, there will be some more differences. But at the species level, there will be different species

that may be affected by the xenobiotic. So we cannot see, at the very fine level, we have commonalities. But at the higher end, we have commonalities that we can find.

Then also like if we look for the short-chain fatty acid or such kind of metabolites, we can find some commonalities. Okay, like bacteria are involved in that metabolism. So that would be one of the things.

DR. DA COSTA: In some regards, as we look at these datasets, they remind me a little bit of genomic datasets where you also have a tremendous amount of data. Some things go out. Some things go down. In the end, sometimes you can pull changes in certain genes or genes associated with this or that, which is what they are also attempting to do here. So definitely not an easy analysis to conduct.

DR. SUTHERLAND: I think it means we have a lot more work to do in the microbiome before we understand really the impacts. That is kind of actually what I am trying to help figure out is where we need to go next.

I really loved the presentation. And I really liked that you had that phased approach concept at the end there. Part of what I am trying to figure out from my end, from toxicology studies, is what would be the best next step for us.

So should we consider when we do tox studies, doing a microbiome analysis? And should it just be, should we start with fecal matter? And maybe, hey, do we see a change in that pathogenic, non-pathogenic ratio that you were talking about? And if so, maybe we should do something further. So at study N, we actually take ileal tissues or colon or something like that and go further.

Is that kind of the proposition that you have right now? You listed a lot of potential further ideas. I am trying to figure out what would be the best next step to help at least the NPT in its tox assessments. I am just wondering if you kind of have a feel for that.

DR. KHARE: If it is a long-term study, I will say yes. Fecal sample definitely is good without sacrificing. You can assess, okay, where that tipping point is actually happening.

But then, that may not be really a true reflection what is happening at the ileum level. So you need to then decide at the ileum level what is happening. I have presented so many results, but there are some results that are showing clearly that at fecal, there is a different kind of bacteria. But that ileum, there is a different kind of bacteria. And those bacteria can be correlated with the host responses also.

For one of the studies, I think in arsenic, I have provided the data results both for the fecal, as well as ileum associated bacteria. And if you see the name of the bacteria, they are different. But they are doing the same function. Like I showed you, okay, there were changing like one was degrading mucin. Another was playing again at saccharolytic bacteria was there. So the first layer of the protection was affected due to the presence of this bacteria.

Then they were also bacteria that were involved either in the gastrointestinal tract permeability changes. But those two bacteria were different. So like whatever immune responses happen basically, it is due to the ileum or like whatever is present inside or whatever is adhered to the mucosa. So I will suggest, yes, fecal will give first a snapshot that we should look. But wherever the change occurred, you have to go back to look for the full profile how it will be affecting other parameters also.

DR. SUTHERLAND: So then it almost sounds like we should continue with some of the paradigm that you have actually been looking at. So let's pull some of those animals down at certain stages, perhaps when we pull biological samples or something like that, and collect.

Then I have another question. If fecal is only really going to give us maybe an idea that something is

happening, and we really should look at other tissues, you focus mostly on the ileum and the colon. Do you happen to know if anyone is actually looking kind of pretty much at the whole intestinal tract?

Right now, it sounds like you are saying that the ileum actually gives the best predictor of what might happen in the host. But what about other regions of the GI tract? Have people looked at them, or is it really that the ileum is the best place?

DR. KHARE: So people have started looking actually ileum and colon. And also the cecum. Cecum is basically like powerhouse for all the metabolites. People have started looking. And some in the study, we have collected cecum tissue also.

So yes, it is quite like challenging. But yes, we are trying to address like one-by-one how we can get like to the main point basically. And even for the fecal, so when we have done analysis for ileum, colon and fecal sample, and it is with one of the studies with the arsenic we have done, and I haven't shown the results here.

So what we have seen like these were the six animals. And all six animals, the bacteria that we are looking, it should be present or absent in all those six animals. And we can see transition, something that is initially present in ileum, colon and fecal sample.

Something is only presenting fecal sample. And then we can see, due to the arsenic exposure, how the transition from ileum, like it is now not present in the ileum, and it is only present in the fecal sample. Earlier, it was not present in the fecal sample. But due to the exposure of xenobiotic, it is now released to the ileum. And the ileum is devoid of that. And that played a major role into the homeostasis. Those kinds of studies are also --

DR. THURMOND: Scott Thurmond. You are probably the only one right now I am aware of who is doing this type of work. It is groundbreaking in a way. And coming from CFSAN where we worry about what goes into our food supply, we have artificial colors, we have natural colors, we have just about everything you can imagine, we have no idea what the impact is on a lot of these products on the microbiome.

And when you are dealing with humans, we all know there is a wide range of variability in microbiome across humans even within cultures, within geographic areas. We have got a long way to go, but I think this is a good start. I think you have got a lifetime of work ahead of you.

DR. BELAND: When you show these like with the arsenic, bacteria will be down. All of sudden, it will be skyrocketing high. Are all the animals behaving the same?

Or is there one animal that is sort of driving this response?

DR. KHARE: Most of the animals are behaving in the same way. Yes. And that is why we have done like the analysis. There is a different way of analysis. So we have used the statistics tool in which we are taking each of the individual animal and then output responses. It is based on whether it is present in all or not present in all. Like a contributing factor from each member.

DR. CARLSON: First I have a comment and maybe a caution here. We need to know what is happening in the context of all of these things. But we need to take a step back and determine if we are just seeing a change as the microbiome changes, or if we are seeing a toxic response to something. And we don't really know how to define that. I think we can all agree that we don't know how to define that yet. That is part of why doing these things is important.

But also, we need to continue all the basic research in the microbiome that is going on across the world because we don't know mechanisms. FMT for C. diff is used constantly, and we don't know how it works. We don't know how the microbiome is affected. And so we need to get at this. The idea of a tox profile for every new drug, we don't require anyone (audio garbled) because we don’t know

how to interpret it. A change does not mean a negative change.

DR. SUTHERLAND: That is why I was asking specifically about the study with the microbiome changes and then the eventual tox effects. I am wondering if that is the path we need to take. I understand we need the three Rs and we need to look at the in vitro methods and stuff.

But I think in the beginning of fields like this, when we want to understand what is going on, and we just don't want to see, well, we have got all of these genes going up and these genes going down, or bacterial strains. I would actually like to see if we could correlate it to a tox effect. And then, does that translate to humans? I am just wondering if that is kind of --

DR. CARLSON: Correlations would be great. Everything we have in the microbiome, for the most part, is an association. There is one arsenic paper that came out recently where they showed a direct link (audio garbled).

I have a question about the arsenic in general. You see shifts where you are losing anaerobes and you are seeing proliferation arrows. Do you know if you are seeing changes in oxygen? Is the arsenic causing some damage in the gut that is changing the oxygen levels? And what you are actually seeing is a side effect of that damage?

DR. KHARE: Probably. I have that, but we haven't done that in -- that was one of the why it is like anaerobic isn't affected so much.

DR. CARLSON: Well, we know when you give antibiotics, the oxygen levels go up. That can be important for various conditions too. I wonder if that is what --

DR. KHARE: We haven't with that. But probably that is something in future studies we can include.

DR. DA COSTA: Would you care to share some thoughts?

DR. CERNIGLIA: The first question in terms of adverse outcomes, I think you have to consider four things. One is the transitory, as was just mentioned. Then you have established, which can certainly happen, and that is a problem. Or it could be developmental, as you were talking about the tipping point, that window. And then more importantly is trans generation. So when you think of exposures, you have to think of those four things. That is the first thing.

Also, I think to get at a question that was asked, both Goncolo and Vicki asked, and think you have to determine, when you are designing a study, it depends upon the question that you are trying to answer. For example,

the point that Paul just made about the arsenic study, it is a very good study. It is an outstanding study.

It proves one of the points that Dan was making on the role of the microbiome, both productive and methylation reactions. In that particular study that Paul Carlson was just talking about, they used the mechanistic approach. They used the germ-free conventional model and antibiotic-treated animal. That would knock out the bugs. And then they also did a gene knockout of that arsenic transfer rates that Dan was talking about when he showed that schematic.

So if you are going to look at the role of the microbiome and arsenic toxicity, it depends upon the question. And that particular question that they addressed was really nice because it touched the basis of the role of the microbiome in the detoxification of arsenic. That is an example of, depending upon the model. So I think you need to have some mechanistic studies, as Paul pointed out.

The other thing I think is very important, and I do a lot of risk assessment. I just got done doing pesticide risk assessments and I have seen a lot of data. And again, I think this was the point that was made. A lot of times, you do see population changes. With all of our greatest techniques that we had metagenomics you could see

changes. But it is a biological change or is it (audio garbled)?

And most of the time from the look of these studies, the animal studies, they never measure. Even though you might see the metatranscriptome and you see a proinflammatory response or something like that, no one goes back to see indeed if there is a clinical effect. I think that is another design that needs to go into these kinds of studies. If you really want to do a study, it is a team approach. All of these particular studies integrated this approach.

We have mentioned samples of working closely with Dan and Mary for both arsenic and aloin. But I think in any study, what we need to do, for example, in triclosan, that is a study that we are doing with Vickie. But the major component that is missing in this study at this point is that triclosan, as you know, is metabolized both in phase 1 and phase 2 metabolites, like 13 or so metabolites that have been identified.

That is going to be very important to know if you are talking about impact on the microbiome, are these metabolites not. Arsenic we know has metabolites and aloin we know the metabolites. But it is important to do that because we really don't know in most of the studies this whole bioavailability issue, that these things really get

into the gut. We did determine that in the study with Dan. But typically, we don't know what really gets there.

And that gets into question that Vickie asked about what do you really look at? I think it depends upon the compound. Some are absorbed differently. A lot of it never gets to the intestinal tract. It gets absorbed in the small intestine, not the large intestine. So again, you have got to look at the classic chemical. You have got to look at the mode of action. And all of that plays a role. We are thinking through all of this.

Sangeeta knows everything I just said. And so we are working together to try to maybe come up with some studies. So for the future, I think we need to carefully consider in terms of really what do you need, the types of studies that could be done.

What was not mentioned, which I think one of the more exciting areas now is this human gut chip microphytic devices. And there are about five or six out there. There is just not one. We talked about the one here that we are working on within the agency.

But actually, internationally, there are chips in Germany, there are a lot of chips that have been out there. It will be interesting to evaluate. All of these models have advantages. They have the challenges. They have their limitations. There is a lot to be done. I agree.

It is a lifetime. But then again, we have been working in this field a long time. This is not new.

This is, the role of microorganisms testing xenobiotic metabolism has been known for many, many years. So I think we just need to focus what information we really need and how can we get at it. I think we can do it.

But we have got to do the right models. There is all kinds of variabilities and across animal studies. As another point that really needs to be made. I really want to emphasize this because I am a critic.

Sometimes like now how informational is the rat microbiome compared to us? Very, very different composition. So you have to be careful when you do these extrapolations. Unless you are doing point studies where you can do that. So I think it is important to do that.

Just one other point that I would like to make is I think this is a key point for developing studies. I think we need to look at subpopulations in our studies of microbiome. By that I mean if you are doing a study with arsenic or something like that, you could look at a colon model, animal model study. You could look at an IPT model study and other disease models to see indeed if that xenobiotic exposure really exacerbates the disease.

In other words, is there some kind of clinical, whether it is obesity models, whatever it is. So then you

can see this because I think if you just do xenobiotic exposure, you are just compounding, you are just looking at population changes, even cultural changes, the signal to noise ratio is not that (indiscernible word) besides that signal that you get versus the background noise, and by the background noise, I mean all the diet and everything else that you have, the fluctuations that you get in your microbiotas. Another way of looking at it too in designing studies, what you don't see as often is subpopulations and how this exposure could exacerbate. So anyway, I have a lot of ideas.

DR. CARLSON: Just to comment on the relationship between mouse and human or rat and human. I really think that what Sangeeta is doing is the way to get at that. It is functional. The strains are going to be different. The functions are going to be simple. So if you look at whole meta genomes or metabolites or other, the best way we can make these models useful. And we really need to ask it is a 16S.

DR. CERNIGLIA: That is true.

DR. THURMOND: Quick comment here. Bolus dosing is great. You get an effect. In CFSAN, we are more worried about long-term feeding studies, what is going on, whether or not if you get a xenobiotic to a mouse, to a rat

or even to a human over a long period of time, what is going to happen with the microbiome?

Maybe at exposure levels that are more representative of what we eat, what levels we eat at. Over time, what happens? Can we monitor changes in the microbiome? Does it recover over time? That is something we would like to see developed.

DR. WALKER: So from a regulatory, can you all see a time where an assessment of the microbiome is like any other system that you might get a report for as part of a package? And how would you do that? And how would you look at the value information? And what would you be looking for because I think this is kind of what we got into this originally was to put together a set of compounds, some of which we know are bacteriostatis and bactericidal and some would jump. But which require good metabolism.

This dataset is quite a robust dataset for starting to ask those questions of what you might want to be asking. What would be the level of evidence to call something as this caused intestinal dysopsis, microbial dysbiosis. Had a tweet, we saw a change, or this was something of concern. Because what I was hearing was essentially three different areas.

The role of the microbiome in kinetics, metabolism, and the compound. Coming from a chemical point of view. The role as a system that could in itself be disturbed. And what would that look like. Where the consequence of that is something that we are concerned about. A reviewer within FDA would look at it and go, oh, I am bothered by this. And you describe XYZ, ABC of different levels of information that is valuable.

I am hearing functional is useful, phyla is useful, the in the weeds and genus and species is probably not that useful. And then the third one is the role of explaining the effects that we are seeing in a rodent in terms of its toxicity. Like the aloin story, that is essentially a mechanistic story there.

You have got clearly these three arms. But I think when we walked into this, we came in with the second arm. We kind of know the impact on good metabolism. We know that there is a role there. And we kind of had a hint. We knew that some kind of analysis might help us with mechanisms from the aloe vera because we looked at small chain fatty acids in that study.

I think you have shown, oh, it is probably a direct hit on the bacteria. I think you are creating a little mode of action there. And the question there would be is that translatable to the human situation. Do you get

a similar situation. But it is this middle ground, the microbiome as a system that is perturbed not in those other two angles, but in and of itself.

A change is functionality something of concern. Separate from its role in metabolism, separate from explaining through some other chemical intermediate or something like that. Does that make sense? And so I am kind of interested to hear from the regulatory folks who can they foresee a day when they -- and if so, what would that look like because that is kind of what we want to hear back. We want to hear what is it that you want to see that would help you go, yes, I am concerned about that.

DR. CARLSON: The issue is we don't know what these profiles, what it means in terms of health. This is a starting point. We see the changes. But people who are researching IBD or others, so many things have been associated with the microbiome. But until we know what specifically is responsible for the phenotypes that we are seeing, it is hard to make a call.

And one of the concerns that I always have when I see a new paper come out associating X with condition Y is that it still just an association. It makes me nervous to think that we are going to make regulatory decisions based on associations that we don't really that is causing it. And so I just don't know that we are there yet.

That is why we don't ask for these things. I know I have been consulted a couple of times from CDR where they have sponsors in data saying their product protects the microbiome. We don't know what that means.

And so we are not going to get a labeling claim based on protecting the microbiome yet unless you have a functional readout. So lower incidence of C. diff following an antibiotic, following the use of an antibiotic, something like that. And until we can advance the basic science to get the mechanism in line, these things are associated with these conditions it is hard for me to imagine making a recommendation based on it.

DR. SUTHERLAND: I am kind of curious. Would it benefit what Scott said earlier where in the longer-term studies where you are doing dietary exposure, if we make sure that something is added to it. You haved an arm that comes off to see if there is a recovery period. Would things like that really be much more informative to you? So if there is not a recovery, you know there has been a permanent change to it. Does that help you? Or is it more like that just adds --

DR. CARLSON: There is always going to be a recovery in the microbiome. It is amazing how quickly it bounces back. And we get re-exposed to things. So where you don't like to think about the fact that you are

ingesting small particles of feces all the time, but you are. We all are. It happens. So we are constantly getting (indiscernible word) in animal studies, it is even more so because (indiscernible word). So that makes it even harder. So there is always going to be a bounce back. I think one question is what is the delay in bounce back. So how much damage is there.

And while I think for the basic science, the research, the functional readouts are going to allow us to interpret the results more, especially comparing the species. I don't think we are there yet to start asking sponsors to do that because it is expensive.

You can do a 16S sequencing run for $40. It is a lot higher; it is $500 or so to do a metagenomic output. And the analysis is much harder. And so I just don't see. This is another instance where if we start requiring this too soon before we can interpret it, we could really hold up drug development.

DR. CERNIGLIA: (Indiscernible - off mic) if we can really get the biomarkers to get at this depending upon what the exposure is. For example, in cardiovascular toxicity, there was a TMAO biomarker that was out there. That is an example where you could point to. With PCBs, another biomarker where the effects on the microbiome was bile acid metabolite. So again, you can target things.

There are examples out there, but not many. That being said, that is an area where I think we could play a role at NCTR.

DR. WALKER: So, Sangeeta, you got six or seven agents. Just building out this database. The more that we know where we have got toxicity from different systems. I know that would only be associations and correlations. Does that at least start to get us down that road of, okay, we know this does this. Have studies in different target groups of like we know these do this type of toxicity and we see this. We know this and we see this.

Is that going to be helpful to the regulatory centers? Or are we going to need more in the weeds, mechanistic information that specifically this leads to this, irrespective of the agents, as to when those functional statements come out, you can make that link. It is with the direction of where you put the best effort.

DR. THURMOND: We need to understand the metabolic function of the microbiome in terms of products, for instance, FD&C colors, which are artificial colors, what happens to it? What is the ADME that the microbiome takes part in, in either toxifying or toxifying these products.

That is our critical concern. What gets into the circulation after the metabolism takes place in the microbiome. You wanted to know where we are going with

this. I think that is pretty much what we need to find out.

DR. CARLSON: We have been talking about do these things do to the microbiome? The microbiome also does things to the drugs. That is the point, and I don't always think about that because that is not (indiscernible word). There is certainly a lot of evidence for the microbiome causing breakdown of drugs into toxic forms. I think that is another thing that we need to know.

(Inaudible comment off mic)

DR. DA COSTA: Thank you very much, everyone. And Sangeeta, it looks like we are going to keep you in the payroll for the foreseeable future. Anyway, so all of this talk about recirculation of fecal matter opened my appetite.

So let's reconvene at 12:45. Thank you.

(Luncheon Break)

AFTERNOON SESSION

Agenda Item: NIEHS/NTP Updates

DR. SUTHERLAND: I would like to introduce our newest postdoc at the National Toxicology Program, Mimi Huang. She is one of our brightest postdocs here. She is going to give us an update on dibutyl phthalate.

DR. HUANG: Good afternoon, everyone. I hope you all enjoyed your lunch and didn't think too much about the microbiome.

My name is Mimi, and I am the current study scientist for dibutyl phthalate. I will be giving an update on the two-year cancer bioassay that we have done on this compound.

So start with a little bit of background. So phthalates are primarily used as plasticizers int eh industry, so making hard plastics more malleable. Although some of the lower molecule weight phthalates, such as DBP, are also used in a variety of other applications, such as the manufacturer of latex adhesives as solvents in personal care products. It is also found in some cosmetics, such as nail polishes.

DBP has also been detected in some pharmaceuticals and it is used as an excipient to modulate gastrointestinal absorption. Then it is also found in a lot of food packaging materials, a lot of the

nitrocellulose, these ecofriendly, plant-based sorts of plastics.

So the primary root of exposure to humans of DBP is through the food. So the phthalates aren't actually bound to the plastic materials, and they migrate from the food contact materials onto the food. And so it gets ingested through that.

There is also some exposure through the dermal and inhalation routes. Primarily through occupational settings, such as manicurists working with nail polish or factory workers in these industries.

So once DBP enters the body, it is metabolized pretty quickly into monobutyl phthalate by a lot of the gut, and then it is excreted primarily through the urine. The primary toxicity associated with DBP and other phthalates is what is termed as phthalate syndrome.

This is characterized by malformations of the male reproductive tract, impaired reproductive development. This is observed within utero exposure to phthalates. And what has been seen in the rodents has been hypothesized to be similar to what is observed in humans and what is termed as human testicular dysgenesis.

And so as a result, a lot of the regulatory values are based off of these developmental effects. I have listed some of them here. As far as FDA guidance that

I haven't found a risk assessment for DBP specifically, but there is one for DHP.

And then I also found that it was not recommended for use as an excipient in one of the guidance documents. So there hasn't been any cancer evaluations done on DBP largely because of the lack of adequate animal studies. That leads to the rationale for undertaking this chronic study because at the time of the study design, there was insufficient data. And even now, surveying the literature, there hasn't been any new cancer studies in animals of DBP.

Then another rationale for this study was kind of when the study was designed, it was designed in the context of other phthalate studies going on. And so the thought is that perhaps we can perhaps evaluate the toxicity of phthalate mixtures using the data that we generate here.

Some design considerations that went into the study design included the use of perinatal exposures and largely because of the known development toxicities. But also due to the widespread exposure of the population to phthalates and DBP. And so it is quite likely that pregnant women would be exposed.

And then as I mentioned earlier, the primary rate of exposure to DBP is through the food. And so we conducted feed studies in our animal studies to emulate the human exposure.

As far as dose selection goes, I have highlighted some of the key findings from our sub chronic studies that we used to design doses for the chronic study. And these are reported in our NTP tox report 30.

So on the rat side, I highlighted the perinatal DRF because we included the perinatal exposure in our chronic study. And as you can see, the doses are listed here. And we saw high mortality of pups at the highest dose at 20,000 parts per million. And there was a slight decrease in the number of live pups per liter and the pup bodyweight at the second highest dose of 10,000. And so that was selected the top dose for the rat perinatal chronic study.

As far as the mice go, I have included results from the 13-week study. And survival was generally unaffected up to the highest dose. And there were some decreases in body weight at doses greater than 5000. But again, these are within 6 to 15 percent of the control. And so 10,000 were selected as the top dose for mice as well. And then because of the known developmental effects, we included a low dose of 300 parts per million in the rat study to kind of cover that broader range of dose.

So there are the study designs for the rat using the Harland Sprague Dawley rats and then the B6C3F1 mice. As I mentioned, it is a perinatal study. So we had

pregnant dams consuming the feed from gestation day 6 all the way into PND21.

We took that animal at two different time points, at BD18 and PND4, and analyzed both the maternal plasma and fetuses or pups. And then after the pups were weaned, they continued to consume the same feed that their mothers had consumed. For another two years for the full necropsy and histopathology was done. And then for the mice, it was just adult exposure for two years. And then then necropsy and histopathology.

So because the DBP is being administered through the feed, I just wanted to give you an idea of what the actual chemical consumption was. So this is factoring in the food consumption. So you have the different doses, and then different study phases here. And you can see that at the lowest dose, 300, the average per day. Consumption is lower than the current NOAELs used to make those regulatory values. And so we are kind of addressing kind of covering that space.

Both male and female consumptions are similar in both the rat and the mouse study. And the mice are consuming around two times more DBP, even though they are given the same concentration of DBP in the feed. That is likely a product of higher food consumption.

And then here I have listed just the human equivalent dose of some of these oral doses using the EPA dose metric adjustment factors. And given what is estimated human DBP consumption is around 1 to 10 micrograms per kilogram per day. And this is in milligrams per kilogram per day. So it is around 10,000 fold higher. I also note that this decimal point is off. It actually should be 15.6 and 14.7.

So I will start off with the results from the rat study. This is in our perinatal phase. We didn't see any differences in mortality or dam body weight during gestation or lactation. And there is a table of various living parameters, as well as body weight. You can see that there are really no significant differences in gestation length, liter size, sex ratio. And there is a slight decrease in pup body weight. That is about 88 percent of control.

These are the biological sampling results. This one is taken at GD18. We have dam plasma in the solid bars. And then amniotic fluid and then fetuses. You can see that there is some gestational transfer of MPB.

Then I have also listed the human MPB, maternal plasma concentration. And again, these are much lower than we are seeing in the animals.

This is the biological sampling data, PND4. And again, dam plasma in the solid bars and then the average of the pups in the white bars. And again, you can see that there is some lactational transfer occurring.

So here we have a survival and body weight. There were similar survival rates. And there were no treatment related at the clinical observations throughout the whole study. This is during the chronic phase.

And then it is also showing the body weight curves for the males and the females on the right. And giving an idea of what the differences from control of the highest dose group.

Listed down here are the terminal sac body weights of both males and females listed as percent of control. You can see that there is a slight decrease with increasing dose. And the values at the top dose are significantly lower.

So we went onto histopathology. The primary gross lesions that were observed were restricted to male reproductive organs. You can see that there are a number of organs that are small or not present. And then evidence of nondistended testes in the abdominal cavity, also greater than 20 millimeter gubernaculum length. And again, a lot of these instances are occurring in that top dose group.

So a lot of these gross lesions, they were microscopic lesions correlated with the gross lesions. So showing you the significant ones here. We have hypospermia in the epididymis. And that is statistically significant at the highest dose. And I will say at the highest dose, the pathologist graded it as the highest severity. So there is essentially no sperm in the epididymis.

See that there is decreased secretory fluid in both the prostate and seminal vesicle. And then various and non-neoplastic lesions in the testes. Then I have included these two, the two at the bottom, because we have seen that in other reports of in utero exposure to DBP. It is interesting that we see it here as well. And again, the incidences of these lesions are significant at the top dose. We also see some non-neoplastic lesions in the pituitary and in the liver. And this is found in both sexes.

As far as neoplastic lesions go, we only saw statistically significant treatment-related trends in two different types of lesions. The first one is in the pancreas, carcinoma or adenoma. This is only in males. Then a statistically significant trend in the uterus.

Again, there were no significant increases. Then if you notice, I have given the historical control range. And many of these incidences fall within that range. So

just summarizing the results from the rat study. So we saw minimal differences during the perinatal phase in dam body weight, littering parameters or pup body weight. We did see some gestational and lactational transfer of MPB.

And there are minimal differences in survival and the body weight over the two years. I listed some of the target tissues. And then we only saw treatment-related trends in neoplasms in the pancreas and uterus.

So moving on to the mouse study, again, this is just adult exposure for two years. There are no differences in survival. And then I have shown the body weight curves, males on the left, females on the right. And you will notice that the difference from control of the top dose group is a little higher than what we saw in the rats.

And because there weren't any treatments related clinical observations, that is the reason why we kept these animals on for this study even though they had much lower body weight. Then again, I have also shown the terminal sac body weights down here.

As far as histopathology, there are some non-neoplastic lesions observed. I have pulled out the ones related to the male reproductive tract as a point of comparison between the mice and the rats. And so you can see that again that the top dose group is where we see a

significant increase in incidences. And then the mice, we didn't see any treatment related increases in neoplasms.

So this slide just summarizes what the results from these two studies. And again, there were no effects on living parameters. We did see some gestational and lactational transfer in the rats.

For both rats and mice, the male reproductive organs were affected, and these occurred at the highest dose. And then there were no statistically significant treatment related increase in neoplasms in both the rats or the mice after two years.

We just had our data review for the study last week. And we are working on getting those tables finalized and the report written up. And we hope to get this into peer review by early next year. I will take any questions. Thanks.

DR. DA COSTA: Thank you, Mimi. I am curious in your introduction, you alluded to that one of the drivers to initiate this study was the possibility of generating data to conduct a read across exercise. What other phthalates do you have as the NTP study that might enable such a read across? Do you have any?

DR. HUANG: We have other phthalate studies. So DHP and then I think butyl benzyl phthalate. I don't know if Nigel can elaborate.

DR. WALKER: DHP is one that we have an ongoing technical report coming out. And diisobutyl phthalate AIBP. The whole reason this was initiated, if you remember, back in the late 2000s, early whatever you call the tens, FDA had a big interest in phthalates. This was part of the phthalate initiative. We had lots of in vitro work and all that kind of stuff. This is one of those things that was one of the last ones out of the gate out of that.

DR. FITZPATRICK: There are a couple of the phthalates on the list. Next was the toxic compound. Is this one of them? I know CDER and CFSAN agreed to help EPA work on those reports. We can pull you in if you want.

DR. BROWN: Just a question. You probably said it and I missed it. Where did the human maternal plasma concentration come from?

DR. HUANG: There have been a couple of studies done primarily in Europe, or the Sweden, Finland type countries. So I took it from those human studies. They are looking at mom plasma and the corresponding, I think they had cord blood too.

DR. SLIKKER: I was curious about the lactation exposure. Were those values what you expected? And did you directly expose the pups, or was it totally through lactational exposure from the mom?

DR. HUANG: So the chemical was being administered through the feed. I would imagine that all the lactational values that you saw in the pups was from the mother.

DR. SLIKKER: How did those exposure levels during lactation compare to levels from the literature or levels that you maybe would hope to obtain? Were they in the same ballpark or were they lesser?

DR. HUANG: I know from human studies where they are looking at the amount of MBP in breast milk compared to plasma, the concentrations are much lower in breast milk. I don't know if there are any animal studies looking at that. So I don't know if there is a point of comparison for that. But as far as like humans, the concentration is much lower in breast milk. I can't say what fold. If you want to know, I can look it up.

DR. DA COSTA: Thank you, Mimi. So next we have Dr. Sutherland, who is going to give an overview of ongoing activities at the NTP.

DR. FITZPATRICK: I have a question on the botanicals. Do we put that under the botanical?

DR. DA COSTA: Yes.

DR. SUTHERLAND: So as Goncalo said, I will be giving you a very brief update on a number of a chemicals that are ongoing at the NTP. The majority of these chemicals are more in the final phase. But if you have any

questions, I have listed the study scientist for each one of them. And the chemicals that we are going to cover today are a couple of botanicals, boron, 4-MI, antibacterials, analgesics, HIV therapeutics and the sunscreens.

Now, for the botanicals, we have two of them that Mimi is actually the study scientist for. The first is resveratrol. And there was concern associated with this one because it is a dietary supplement that has widespread exposure through foods. And people are using it for therapeutic or in conjunction with other therapies.

So we performed a sub-chronic and immunotox and a reproductive assessment, continuous breeding studies. And for each of these studies, they are in the reporting process and will be reported out mid next year. So that data should be coming soon.

The chronic study for resveratrol is actually in pathology review. And the data from that study is actually expected to be released in 2020.

Mimi also took over the black cohosh program from Chad. And as many of you know, this was a concern associated with this because women of childbearing potential or in the perimenopausal period were using this botanical for premenstrual or menopausal symptoms. We also did a reproductive continuing breeding assessing on this

one as well. That study is in Pathology Review. Plans are to report that as a manuscript by the end of 2020.

We also have a chronic study for this that is in the pathology review process. This should be reported out as a technical report by the end of 2020. And I should mention at this stage, there are several different reporting processes that the NTP uses.

We have got the technical and the tox reports that you are probably most familiar with. But then there is generally an encouraging to go ahead and produce the manuscripts for a lot of this work, especially in some of the smaller studies or the one-off studies that we probably won't be needing to develop a tox report series for such as the RACDs.

There is also sufficient similarity evaluations ongoing for the botanicals. I will talk about that just a little bit more in a moment.

The other botanical that I wanted to mention was echinacea. Kristen Ryan is the study scientist for this botanical. And this one is primarily used for stimulating the immune system. There were concerns associated with this because of use during pregnancy. And of course, there was a lack of evidence to support the safety and efficacy.

But another concern associated specifically with this botanical was the test article selection. So we

really wanted to ensure that the selected chemical was very similar to what was on the market, free of contaminants and what was considered pure was based on the markers if we had those available. And so I will mention the test article selection again in just a moment.

But for echinacea, we had a number of studies. Two of them being 28-day repeat studies with immunotox endpoints, one in mouse and one rat. Both of those are complete. The mice report is on file. And then rat in life is complete, and we are waiting for the lab report.

We also performed a modified 1 gen reprotox evaluation in rats for this compound. And the end life for that one is complete, and we are also waiting on a lab report. That is expected in the next year or two.

We also have an invitro immune stimulation assay, and that study is complete. And again, we are waiting on the lab report. So we have a lot of stuff coming up. Just if you can stay tuned for another year or so, we will have a lot coming out for you, especially in the botanicals.

Now, the botanical program, as most of you probably know, is being run by Cynthia Rider. And one of the main goals of this that I wanted to bring forward was that she is working to develop a screening level approach to basically incorporate both chemical and biological similarities essentially to inform test article selection.

And this would allow us to extrapolate the tox findings from tested material to other nominally related products. So essentially, any particularly chemical or test article that was closely related to the test article that we chose to test might actually have some similar or sufficient similarity case for assessing predicted tox effects in that particular test article.

And so to this end, we actually performed a series of case studies evaluating the sufficient similarity of a number of botanical products, and comparing them to the tested test article at NTP. We plan to continue the testing for this, as well as refining the methods, et cetera. But we would really love any feedback from the FDA on any potential botanicals that might be interested in incorporating in this.

A second goal for Cynthia's botanical program is essentially enhancing the botanical safety toolkit. Essentially, we are trying to build bridges between what is our current gold standard animal safety testing studies and the alternative assays. And she has got a number of key focus areas here, everything ranging from chemical analysis, priority endpoint systems, all the way to data analysis.

And they have already signed an MOU between HESI, FDA and NIHS. And we are actually actively looking at the

funding process for this. So this is something that is upcoming for the botanical program.

Now, for our next program, boron, this one is being run by another postdoc at the NTP, AtLee. And it was nominated by the State of Minnesota based on concerns for infants and neonatal sensitivity to boron, especially since boron is found both in the public and private water systems, and also present in infant formula.

There were no studies available to essentially evaluate anything associated with direct postnatal exposure for boron. So AtLee designed a program where we evaluated the developmental and reproductive toxicity following both prenatal and postnatal exposure to determine whether or not the prenatal and postnatal exposure was actually a more sensitive exposure window than just prenatal alone.

We found that there were dose-dependent increases in plasma boron concentration at each of the timepoints. And basically, the study results showed that there was a dose related increase in mortality and an increased incidence of umbilical hernias. And these umbilical hernias did resolve themselves in the longer-term study. But we also saw significant lower pup body weight at the 12 and 16 milligram per kilogram doses, which were the two highest doses tested in the longer-term study.

The body weight also had partially rebounded during the sub chronic exposure period. But at the doses we tested, we actually didn't see any evidence of male reproductive toxicity in adult males. This has been put in the literature that reproductive toxicity for boron was of concern. But at what we tested in the study, we did not see this.

The endpoints for this particular study are two different manuscripts, the boron dose-range finding study is predicted to come out fourth quarter 2019. And then the sub-chronic study is looking at second quarter in 2020.

We also had the 4-MI program that is run by Mamta Behl. Now, this was to assess the potential reproductive and developmental toxicity of 4MI because the general population was exposed to this through the consumption of caramel-colored foods. And there is a possibility of some occupational exposure.

We also performed an RACB study with this one as well. This is actually in manuscript form at the moment and has been sent to the FDA. At the moment, the release date has been embargoed, so we have the opportunity to do some communication pieces prior to the release if the FDA so desires. We need to make sure that communication is kept open between us.

And yours truly is looking at the antibacterial program. Since I have been so lucky with all my programs, we are going to basically kill this one. Mostly because there seems to be a lack of strong stakeholder interest in triclosan and triclocarban because it turns out that some of the public outcry has led to removal of these products. And so the exposure levels aren't as high or at least predicted to be as high.

But just so you know, we did do a dose range finding study with microbiome endpoints as was pointed out earlier. And I think this was a fabulous study. I am excited to get the publication out there. So we are looking at the first half of 2020, once Sangeeta and I have actually have time to sit down and discuss what will go in which particular publication.

For triclocarban, we didn't do any in vivo studies. We have a bit of chemistry work that was done to support the program. So if there is any interest in looking at that chemistry work, please contact me and let me know. But to this date, we are not planning on any publications or anything on that.

So essentially, there are no plans for future or additional work with either of these programs unless you come back to us and say that you are interested.

I have also had the luck of dealing with the acetaminophen project or analgesics program. And I am sure you are excited to hear that NTP assessed the relationship between maternal exposure as specifically for acetaminophen, and looked at developmental and adverse reproductive endpoints in the male. And the reason we did this was it was nominated because there were recent studies that suggested maternal consumption of acetaminophen during particular stages of pregnancy might lead to things such as cryptorchidism.

So the NTP decided to do one study doing a sub chronic type study where we essentially dosed animals during the window of male susceptibility. In this case, rats for gestation days 14 to 18. And then we evaluated pubertal factors.

The end life is now complete for that study. And we are waiting on the lab report that is, fingers crossed, hopefully supposed to come to us. second quarter 2020.

I also have the luck of the HIV program therapeutics. Kristen Ryan is running this program and is trying to toss it in my lap. It turns out I don't know how to run fast enough, so I may end up with this one as well. But Kristin has done a substantial amount of work, especially being in touch with clinicians. They are a primary stakeholder.

Now, for the HIV therapies, NTP receives annual funding from the Office of AIDS Research. So essentially, it is a line item for us to do HIV work. And our current portfolio is essentially testing the combined antiretroviral therapies as during pregnancy.

So essentially, most of the clinicians are very concerned about postnatal effects. If mom is taking an antiretroviral therapy, what happens to her offspring, especially later on in life. So the majority of the studies ongoing at NTP are focused in this area.

Currently, we have two different combination studies. One has tenofovir, emtricitabine and efavirenz done in mice. And then the second combo is abacavir, dolutegravir and lamivudine in rats. The study design is our typical dose during pregnancy and then through weaning, and then taking the animals, the offspring, out for a 90-day assessment.

Now, the dose ratios that we are using are the same that are of clinical use. And as I mentioned, of course, the clinicians are interested in all of those postnatal effects. That is where the majority of the evaluations are.

For our products for this, the range-finding study for combo 1 is complete. The same with the preliminary TK studies and evaluation of individual

compounds and the combination compound. That is also complete. And the sub chronic study is complete. We are waiting for a lab report on that.

For combo 2, we have a range-finder study that is actually ongoing. And then plans for a longer-term study next year.

Now, the reason I mentioned that this program was primarily focused on the clinicians is because they are our primary stakeholder. However, we have a very strong shift at the NTP on what our focus is on things.

And one of the things Brian has challenged us for is looking at the HIV program and asking, is there anything more that we can do. We are currently running guideline-like studies essentially specifically for tri combo therapies. But there is concern from the clinicians, of course, for using these combo therapies say before conceiving or using additional models that may help us answer questions.

And so I wanted to bring this forward to you today because I am very interested in finding out if FDA has any particular areas or gaps that they see in the HIV therapeutics that perhaps the NTP can fill in.

And last but not least is our sunscreen program. Barry McIntyre is the program lead for this program. And I am going to specifically talk to you about HMB today. We

have run a perinatal tox and parc study in both rats and mice. And of course, there is the endocrine disruptor panel that is also being done.

The peer review for this is scheduled for this December with the draft posted on the website. And just briefly, the findings from this study, in the rats, there is equivocal evidence of carcinogenic activity based on the occurrence of malignant meningiomas in the brain and spinal cord. As well as higher incidence of C-cell adenomas of the thyroid gland and higher incidence of stromal polyp and stromal sarcoma in the uterus.

Findings that did not contribute to the call, but we thought we would bring forward, were increased incidences of non-neoplastic lesions of the uterus and the adrenal cortex. And then we noticed an increased incidence of fibroid necrosis of the arterials and intertestinal hyperplasia in the testes.

But there was no evidence of carcinogenic activity in the male or female mice at the exposure levels that we tested. So we did see possible equivocal evidence of carcinogenic activity in the rats, but not in the mice.

We also have modified 1gen technical reports in progress. Now, for HMB, the report is expected the first half of 2020. And for EHMC, the report is expected in the second half of 2020. The respective tables will be

available, or have been available, via the public website. And if you have any questions, please reach out to Barry.

We also have a few manuscripts. One has been accepted for publication. And then the draft one for the endocrine disruptor panel. That is all that I would like to mention to you today.

But as a side note, since Nigel is kindly encouraging me to be part of the IAA, I would like to ask if updates like this are what you would like to see. Or as our discussions with CFSAN yesterday, perhaps seeing some of the stuff at the earlier stages and the designing stages would be more of interest.

So if you don't mind sending feedback, I would appreciate hearing it. Thank you.

DR. DA COSTA: So I was curious about one particular one. On the boron, so you are faced with a nomination by State of Minnesota, that they are concerned about boron. But depending upon which specific species of boron you select, you may expect different results.

DR. SUTHERLAND: We used boric acid in the study. AtLee did an incredible job working with Minnesota in designing the study, making sure they addressed the needs that that particular stakeholder had. And that was one of the questions that was produced.

DR. BELAND: Looking at these triple combinations, the antiretrovirals, why are you doing one study in mice and another study in rats?

DR. SUTHERLAND: This actually had to do with the ADME TK profiles and stuff. I would have to double check what the exact information was on which ones. But when Kristen took over the program, there were certain criteria for evaluating them and trying to figure out what species was best to do this in.

And so we tend to favor rats for a lot of our studies and stuff. But in the case of the first combination, apparently the TK profile looked a lot better in the mice, so we went forward with that.

DR. HEFLICH: Going back to black cohosh, I am aware of the fact that there has been a lot of gene tox data generated. Did that factor into any of these studies?

DR. SUTHERLAND: Can I throw that back at Mimi? I am sure that some of that will be incorporated in the tox reports. But I don't know if they have got separate publications out for that, or if it is just incorporation in the report. Mimi?

DR. HUANG: So the question was if the gene tox is going to be incorporated into the report?

DR. HEFLICH: Well, did it influence the animal studies you have done. Because I know they recently published that black cohosh is an anagen.

DR. HUANG: A lot of it was done in parallel as far as I understand. I think we designed it and conducted it as they were.

DR. SUTHERLAND: We can double check on that to make sure. I thought Christine did a lot of that gene work on black cohosh earlier on. But maybe I am thinking of another chemical. I would have to check.

DR. HEFLICH: There is a lot of data on black cohosh. I think they figured out that it is actually an anagen.

DR. SUTHERLAND: Anything else?

DR. FITZPATRICK: You answered my question. I was just hoping that those were all in your botanical consortium.

DR. DA COSTA: So now we have Nigel Walker. He is also going to give an update on certain aspects of the NTP interagency agreement.

DR. WALKER: We are trying to give you a flavor of different kinds of updates. One was the depth of one of the technical courses coming out. Vickie has given you a update, real high-level on a variety of things that are ongoing. And I will give you more of the more strategic

update for the NIHS/NTP covering the strategic realignment activities we have got going on and the status of the interagency agreement.

Just a reminder that we have been going this past year through quite a refinement of our strategy for what we are doing at NIHS. Really trying to move way from this very just only focused on nominations to more projects and programs and strategic areas.

Where we can integrate across the whole program and the different types of approaches that we can bring to bear on problems. You think traditionally of guideline talks and mechanistic in the court. We are trying to bring all this to bear on these focal areas.

Also have been trying to look at a revised governing structure, so that we can have appropriate oversight to these programs. So that we can ultimately look at the resources we have to bring to bear on our problem, execute in a timely manner and get data back to stakeholders in a timely manner as well.

One of the big things that we have been talking a lot about is translation. One of the discussions we had with CFSAN yesterday was we often have the information out there without context, whether that be exposure context, mechanism context. Translation is kind of something that is front and center of our brains. Brian is really pushing

us on that to really thinking about moving our science into policy, so that people can use it, so people understand the actual translational relevance in humans.

Some of the precision tox information of making it relevant to subpopulations and specific potentially based on genetics of populations. New modes of doing research that might be innovative in terms of high-throughput screening. How do we move those into actual practice? So translation is now something that is cutting across every part of our portfolio.

And this past year has been a very busy year. We started off, since Brian arrived back at the beginning of January 2018, with full review of everything we had ongoing within NTP. Looking at the principles of how we want to do stuff, the governance structures. How our organization is working internally in terms of the people, the other processes we look at.

How we publish. We traditionally have had those NTP reports, as well as peer reviewed manuscripts. How do we get data out? What are different data streams that we can start to entertain? How do we engaged stakeholders differently? Essentially, we are looking at everything that the NIH/NTP has ongoing and how we do it.

Including even down to the structure that we have, be it our scientific structure of our teams come

together, which is both our scientific as well as lay it on top of the administrative structure. Succession planning, bringing people forward to do that cross divisional kind of organization and empowerment. So really, it has been a very busy year being a member of the leadership of like bringing this. So you are seeing probably some changes.

I know that the interagency agreement is part of this. There has been some uncertainties about what has been going on, where is the direction of the interagency agreement. I just wanted to give you this as kind of the backdrop on this is all kind of related to just a total revitalization. And again, a strategic realigning of everything in NTP.

The big thing that has been happening this summer has been we mentioned earlier on last year about the startup of these new health effects innovation programs on cardiovascular health, on revisioning how we might do carcinogenicity assessments, and developmental neuro tox. We came down to NCTR and talked about that, I think it was about a year ago, wasn't it, Bill?

But in addition to these health effects innovations, we have created additional focal areas which are based around exposure-based themes and responsive research themes. Under the exposure themes, we have combined exposure of mixtures. This a major area that we

have invested a lot in over the last 5 to 10 years, and we will continue to focus on.

Consumer products and therapeutics, you saw we have got HIV as an area that we are actually tasked by NIH to be the lead on. But we realize that consumer products runs the gamut from sunscreens to botanicals to a variety of different products.

And the other one with NIOSH being one of our other partners, as well as FDA, occupational inhalation exposures is another focal area because this requires specialized technologies, new approaches. We are trying to bring in some of the new things we are going to be talking about tomorrow in terms of new approaches to inhalation exposures. So these three are our exposure based themes.

Under the responsive research, as you know, PFAS is a key area that all the agencies across the HHS and EPA are actively engaged in. So that is a set focal area. Eventually, that may go away as that gets solved and a new focal area will come back in.

I tend to keep a space open strategically for something we don't yet know. What is it, the known unknowns? We have emerging contaminants and issues as a focal area. So to strategically be reaching out to different stakeholders, see what is emerging and where we should be starting to put emphasis.

And the final one, this is the safe and sustainable alternatives. We are always talking about chasing the whack-a-mole scenario, like always chasing something. Maybe we should start about doing work that will actually help us develop better alternatives from the ground up.

Underlying all this is the scientific cyber infrastructure. We generate a lot of data, as you know. And so making sure we have good cyber infrastructure is important. And also, a specific area to be more strategic about approaches, high-throughout screening, metabolomics. They are often done by different groups in different areas for different reasons. So bringing that as a specific area that we can keep some coordination on is important.

And so essentially within the scope of these programs, the things that we are going to be asking are what problems are we trying to solve, who are the stakeholders, how are they being engaged, how will studies actually actively engage the pipeline of all the different approaches that we can bring to bear on a problem? What are the resource needs?

We have often taken on too many things that are taking too long in the past. And therefore, our responsiveness has been probably not what we had desired. So understanding duration research needs in the products

and how that is going to be communicated is really important. It is part of that active project management, identifying key decisions, milestones. And ultimately, we want to make sure that the information we generate is really impacting decision-making. That is kind of why we are doing this.

That is the background in which the interagency agreement sits. So just remember we have really valued these interagency collaborations. And there is a commitment to continue them.

At the same time, these investments that we do have with our other agencies where, through our interagency agreements, we want to make sure they optimally leverage everyone's strengths. Doing redundant research or stuff that could be done elsewhere isn't the best use of all the talents that we can bring to bear.

And also, because we are going through all the strategic realignment, there is a goal that those investments should actually align with those strategic intents to actually actively address public health issues. We shouldn't be replicating internal stuff. We have said this several times. And make sure we are communicating on what we are doing under these different interagency agreements because we have three or four of them. Make sure that is visible across all partners.

So in terms of specifically the interagency agreement we have with FDA, just so everyone is aware, this is actually supported by NIH's money. There is still sometimes a miscommunication that people think that somehow there is money assigned to the NTP that includes FDA, and that this is FDA money or something. What we give to you in the interagency agreement is NIHS dollars. It is stuff that would go to NIHS programs, but we choose to make that investment because we appreciate and want to use the talents that we have with our partners and particularly with FDA and NCTR.

Currently, that is allocated at 6 million for FY20. As you all know, the project development process is right here. We have these discussions about what are the needs, the regulatory centers. What are your needs for a project area. We ultimately want it to be influential. We review projects as you know every May and November. Ultimately, that leads to the development of protocols, goes through Amy. She sends that to the project officers, and we do a review of that.

The new thing that we have included now is just to seek how these projects interface in with the strategic areas. So again, that strategy is not meant to be a roadblock. It is to make sure there is coordination of everything we are doing, so that there is not duplication

to see how and to make sure that there is governance in these focal areas. Because there is not only our intentional investments, but also the investments that we are doing with our partners.

And then ultimately, that goes through review, protocol review and then final approval or disapproval. This caused some angst last year that the NTP governance group is now part of that. It is actually no different than what it used to be where the protocols came to me, and I would talk to three or four people internally about the review of those. Now it is just a little more formal that we have different internal governance groups.

The biggest news for me, though, is that I am heading off into the sunset in terms of being the project officer. I am not retiring. But Dr. Vickie Sutherland is going to be taking over as the project officer in FY20, pretty much at this meeting.

That is why Vickie was given that update. You probably don't know, but Vickie got her PhD in physiology from the University of Arizona. She was a reproductive toxicologist at Bristol Meyer Squibb. Has a lot of experience. You have seen her sitting at the table. You probably didn't know this but been the study director of monitoring for regulatory investigate studies. Has a very deep background in interacting with FDA in FDA types of

projects. I think she is ideally suited to be taking over this role and has been at the NIHS for the past six years.

I have been 12 years doing this, so it felt like it was an appropriate time to kind of shift and let some of our mid-career staff take over. So I will still be coming to the TSSRC. Just so you know, I am on those internal governance groups.

So the projects that come here ultimately I will still be seeing them. I just wanted to let you know that. It has been a real pleasure sitting next to Paul and then Goncalo these many years now. And with that, I will take any questions.

Agenda Item: Discussion

DR. DA COSTA: So these last couple of years have been pretty complicated when it comes to the management of the IAA because then there were some questions that needed to be clarified. We have been doing that as to, for example, all the money flows into the process, et cetera. That was important to make it clear.

But we are still at the stage where it is still pretty fuzzy. What is it that we should even consider bringing to the table? Because when I look at your three prime interests, which are subdivided into nine sub interests, they are very broad. I could argue that mostly anything under the sun fits there. So this does not inform

me very much about what is in the strategic interest of the program or not.

DR. WALKER: So these were started up earlier in the summer. Each of these has a team associated with them. And each are putting together the strategic plan for them. So part of that will be what we are looking to see. Those strategic plans are going to be reviewed by the leadership team to see if that is the direction we want the organization to go. That is going to be something that we share with not only internally, but also externally. That would give a better sense of what kind of projects and research needs might fall into those four specifically proposed projects.

The other thing is the emerging issues of concern. These are always things that are the classic, hey, something is emerging, we don't know what to do with it. We have always got that opportunity like we have always had of identifying areas where there are data gaps that no one else is going to do that can be addressed. But we want to make sure that there is a group that actually managing that, and it is not just one-by-one. So these strategic plans that will come out of these groups should be informative on that, I am hoping.

DR. DA COSTA: That should shed a little bit of extra light into what we make and bring into the title.

So aren't you concerned that these -- and I know that you argue that there is not an extra level of scrutiny, but it is a much more structured process. So it is going to take longer.

So one of the concerns that I often here from the product centers is how long, from the moment that I identify a problem, that it takes to bring a study to fruition, completion, reporting, et cetera. So I would urge you to think about the process whereby this does not delay much more the process that we have. Because often, being able to respond quickly to that is of essence.

So the other aspect that I am a little bit confused, and perhaps more concerned and confused, was that as per what you just said, and also from discussions, my interpretation was that let's imagine that a product center approaches me and says, look, we are really concerned about compound X, and we would like to study why.

It is my interpretation right now that if this is a study, that you are also positioned to conduct through your CROs that you are not willing to sponsor it in the IAA. But rather, you would be wanting to conduct it yourselves. Am I right in this interpretation?

DR. WALKER: It is a good question. I would say we would look at what are the resources that would be best placed. I mean, if we have got capacity in the CROs that

we currently have, it would make sense for us to maintain that capability.

DR. DA COSTA: That was one that I thought, okay, why should we be --

DR. WALKER: So historically, there have always been things where, in some cases, there have been compounds, they have been nominated to the NTP where the NTP did the studies. And there have been other situations where there have been projects nominated where those studies have been funded through the interagency agreement and conducted at NCTR.

Again, the idea is we should be looking at those areas where the skills, we are leveraging the best talents. So some of the stuff that Dan was doing this morning on the chemistry of arsenic, world-class chemistry, world-class toxicokinetic modeling. There is an extra value added of using the mind trust at NCTR. Using a plain jane 90-day tox study that the CRO can do every day of the week. That doesn't necessarily leverage the best talents. Just to use two extremes as it were.

DR. DA COSTA: Yes, but that goes towards what my understanding was. And I just wanted to make sure that I was right there. So we will be seeing a shift in the paradigms that we will be bringing to the IAA meeting. So we will be seeing things more similar to what

Choufit(phonetic) or Sangeeta have done, and less so, for example, the study that I have conducted on BVL, perhaps a tox study. But if it was a bioaccumulation study, it might fit in. Am I right?

DR. WALKER: It is a change in paradigm. It is not a one size fits all. We are not just the tox testing organization. We have been trying to tell people that for a long time.

DR. DOERGE: Nigel, I understand the commitment of the NIH in general over the last decade or longer to this whole notion of translational outreach. I am a little unclear. I understand the difficulties involved in taking environmental exposures and translating them to clinical style outcomes. So I am sympathetic to a point.

I guess my question is that under the now X demonstration at NIEHS, there was a specific implementation within the DNTP to incorporate specifically this office of health assessment and translation conspicuously absent from any discussion in your presentation today. Can you explain to us how those resources and whatever that allocation is feeds into your programs in general and to the IAA mission specifically. Because I have some examples that we can talk about offline. But more generally, how does that critical goal get met by this specific commitment that you have made?

DR. WALKER: What we have here are the scientific programs that is layered on top of the administrative structure. So the likes of the different offices and the labs and the branches that we have within DNTP are the resources and the areas of expertise that we will bring to it.

In the example of LHAT, primarily that role is evidence integration, literature mining, pulling systematic evaluation of different data streams, those kinds of things. To that extent, a person from that is on each -- the way we have structured these particular governance groups is the day I represented across the whole organization. It is not just all the folks from my toxicology branch or in this one.

And all of the pathologists are here. It is actually a multidisciplinary governance group, so that the principles being what we want to do are within each of these programs. I don't know if that address what you are asking.

(Inaudible -- off mic)

DR. DA COSTA: So we will be learning. Right now, my concern, essentially very soon after I was recruited by the FDA, I became involved in the IAA through the melamine and cyanuric acid project. I have grown to appreciate the value of these meetings.

A crucial element for the value of these meetings comes from the attendance of the product centers. My concern, and I am being entirely honest with everyone here, is in seeing these programs dwindling in the number of compounds or programs that I am concerned whether we can sustain it.

DR. BELAND: You say there is $6 million, but you have also made it clear that there is not $6 million. This happens to be the number for this year. This whole meeting, we have not introduced anything new. This is a total review. Up until now, looking at the program, so I am concerned. Can we even count on $6 million? I agree with Goncalo. I think this program is dwindling. Things we have brought forward never seem to go any place any longer.

DR. WALKER: Like I say, we are in a changing paradigm of a strategic realignment, and that is going to cause some change until we figure out what the next phase on is going to be.

Probably the other news as well is that as you know, our director, NTP director, Linda Birnbaum retired from federal service in October. So we are in the middle of, or I should say NIH is in the middle of recruiting a new institute director. That should take anywhere from 12 to 18 months. And if history is anything to go by, the new

NIHS and NTP director always has an impact on the NTP. And so this is only one change that we are trying to put a scientific strategy in place that may actually even change with that person coming in.

DR. DA COSTA: I understand that cap of 6 million applies to all the interactions with the FDA. Not only the interagency agreement, but also the other one that you have with CDR perhaps with Botanical Consortium, correct?

I don't know if there are any other questions. Otherwise, we will break. Sorry. Actually at this stage, one of the novelties in this TSSRC is that we have also here colleagues from NIOSH. I am very pleased that you are able to join us in this meeting because the other leg in the stool of the NTP. It is always interesting to see your perspective on a common interest. So we appreciate you coming here.

And again, I am reminding everyone, so some of you are not here in the morning. Tomorrow morning between 9 and 11 a.m. in the same room, we will be discussing in more detail, the aid-liquid interface system that not only NCTR, but also NIOSH are using. And it is going to be a more in-depth discussion about validations (off mic) practical challenges. So anyone that is interested in joining us, please just show up between 9 and 11. Thank you.

Agenda Item: Air Liquid Interface Model, Update

DR. CAO: I will give an update on E2200. I thought this would be the last time I talk about this project at a TSSRC meeting. But we didn't complete the project as proposed and are working under the extension. I really want to thank NTP and Goncalo for understanding the challenges of the project and also giving us the time to explore.

I also want to thank our team members, for methodically working out the method to generate formaldehyde vapors. It turns out that formaldehyde is much hotter than we had expected. So we are very pleased to have some interesting data just in time to share with everybody at this meeting.

Again, the goal of the project is to develop a physiologically relevant in vitro respiratory test platform. This task platform has three components. They are the human in vitro air-liquid-interface airway tissue model, in vitro exposure models that mimic human inhalation exposures, and a panel of disease-relevant toxicity endpoints.

As technologies advance, each of these components can be improved to increase the human relevance of the test platform. So we have learned a great deal of information on in vitro inhalation toxicology over the years from

working on various projects. With E2200, in vitro exposure methods for aerosols and vapors, which are proposed under objective number 1, are extensively explored.

As a biologist, I think this component of the project is most challenging and has caused several hiccups to slow down our progress. So far we have completed evaluation of three chemicals starring ortho-phthaldehyde, dihydroxyacetone. We are working on the last two chemicals, formaldehyde and glutaraldehyde. That will be my updates for this meeting.

For some of these chemicals, there is also already a considerable amount of in vivo data. And I think these in vivo data are available assets for not only making the in vitro to in vivo comparisons, but also for designing chemical-specific experimental approaches.

The first chemical I will talk about is formaldehyde. Formaldehyde is a colorless reactant and readily polymerizing aldehyde. It is classified as Group 1 carcinogen by IARC, exposures to high levels of formaldehyde can occur under either the occupational or non-occupational settings. An eight-hour time average, .75 PPM and a 15-minute short-term exposure limit of 2 PPM are recommended by OSHA in the workplace based on sensory irritations. Formaldehyde can be metabolized to methanol

or formate. It is known to cause oxidative stress, genetic damage and is carcinogenic.

So formaldehyde is unique in that it has very low boiling point. It is readily degraded at high temperatures. And neat formaldehyde is not available. These factors considerably limited not only the ranges of the parameters we can play around with the vapor generation system, but also the maximum concentration of formaldehyde vapors that can be generated using the system.

For the validation study, 37 percent formaldehyde aqueous solution was used. Two heating temperatures, 40 Celsius and 50 Celsius and three pumping speeds, 15, 30 and 60 microliter per hour were tested in different combinations. For each combination, formaldehyde vapors were collected 10 minutes and 60 minutes after the initiation of vapor generation. So the goal of the validation study is to generate stable vapors of formaldehyde with the highest possible concentrations

By varying the pumping speed, we are able to generate formaldehyde at different concentrations. The highest concentration that we can achieve is about 35 ppm at the pumping speed of 60 microliter per hour and the injection site temperature of 50 Celsius.

For the preliminary dose range finding study, the ALI cultures were exposed to formaldehyde vapors at 7.5, 15

and 30 ppm four hours each day for four consecutive days. And teacher response were monitored 24 hours after each exposure. And 30 ppm formaldehyde elicited severe cytotoxicity and caused a peeling of the cells after two repeated exposures. This group of the cultures therefore were terminated after two exposures.

Both the LDH and MTS assay were used for aiding the cytotoxicity induced by formaldehyde because we are concerned that the crosslinking property of formaldehyde may confound the results of LDH and transepithelial electrical resistance, also which measure tissue integrity. Significant increase in the release of LDH was induced by 16 ppm formaldehyde after three exposures.

However, the MTS assay, which measures the metabolic capability of the life cells, detected only about 25 percent decrease in cell viability. And there are no changes in tissue integrity based on the tiered measurements. It is very possible that what we saw with the LDH and transepithelial electrical resistance may be the net effect between cytotoxicity and the protein crosslinking. So in this case, we think that MTS assay may be more reliable to reflect the cytotoxicity induced by formaldehyde.

Formaldehyde is known to cause oxidative stress. We therefore measure the intercellular levels of GSH and

GSSG 20 minutes after the first exposure. So the GSH was quickly depleted by formaldehyde. The level of GSSG was also decreased due to the depletion of GSH reserves. And these changes, such as the disturbance of GSH homeostasis, which may lead to oxidative stress. A comprehensive time course measurement on GSH and GSSG will be conducted in the next study.

To corroborate the GSH findings, we measured expression of several proteins representing different antioxidant systems activated by different inducers. HMOX-1 has more diverse inducer compared to these other proteins. And the expression of HMOX-1 was increased by formaldehyde after four exposures. The expression of these other proteins were minimally affected. Which makes sense because these proteins require more specific inducer to be activated. So based on the GSH and the HMOX-1 data, it is possible that formaldehyde has induced oxidative stress in the LAI system.

Persistent oxidative stress may lead to inflammatory response. So in this study, we found this equation of L8, 9, 17 and possibly IP 10 and TNF alpha were induced after four exposures to formaldehyde. All of these molecules have been implicated in the pathogenesis of lung disease. And it is most noteworthy that in animals exposed to formaldehyde, the level of TNF alpha was also elevated.

We then look at the functional changes by assessing the responses of ciliated cells and disturbance of using homeostasis. Exposure to formaldehyde significantly accelerated the feeding ciliated cells. However, the active ciliary points were consistently decreased. All treatment groups at all time points.

And formaldehyde also decreased the expression of three proteins that are implicated in different phases of ciliogenesis. So it is possible that both the function and the structure of cilia cells may be compromised with longer-term exposure to formaldehyde.

To assess the changes in using homeostasis, we included club cell ciliary protein in addition to MUC5AC and MUC5B we charted two proteins we routinely screen in the lab, because of the colocalization between CCSP and MUC5AC and MUC5BB. So the secretion of MUC5AC and MUC5B, but not CCSP, were slightly decreased after four exposures to formaldehyde. And the intracellular expression of these mucin proteins were all consistently inhibited after four exposures. And based on the cilia data and using homeostasis, formaldehyde may have compromised the co-occurrence(?) mechanism of the lung in these cultures.

Formaldehyde is genotoxic. It causes DNA damage and inhibits DNA repair. So in the next study, we will run a common assay to look at DNA damage when the test doses

are finalized. For this preliminary study, we look at expression of MGMT, which is a DNA repair enzyme. Although the involvement of MGMT in the genotoxicity of formaldehyde is controversial.

We found about 30 percent reduction in the expression of MGMT, suggesting it may be involved in the genotoxicity of formaldehyde. The next step, we will look at other DNA repair enzymes that are more relevant to the genotoxicity of formaldehyde.

And the next study we did is to look at aldehyde metabolism. Formaldehyde can be metabolized by aldehyde metabolic enzymes. Most of these enzymes are inducible. So we look at expression of three aldehyde metabolic enzymes that we already have in the lab.

And we found formaldehyde, four exposures to formaldehyde, increased the level of AKR1B10. I am not sure about the significance of the increase of AKR1B10 in formaldehyde metabolism. However, the induction of AKR1B10 has been correlated to squamous cell carcinoma in human cancers, lung cancers.

And also we will look at the expression of ALDH2 and ADH3. Those are the enzymes that are known to be involved in formaldehyde metabolisms. They are more relevant compared to these ones we tested simply because we have these antibodies.

With regard to possible morphological transformations, we look at expression of involucrin and CK6. Exposure to formaldehyde significantly induced expression of involucrin. Compared to CK6, involucrin is an earlier biomarker for squamous cell differentiation. Unrepaired squamous cell differentiation may eventually lead to squama cell carcinomas. And in the animal models, formaldehyde is known to cause nasal squama cell carcinoma.

So here are the major findings of the preliminary study on formaldehyde. I think this preliminary study indicates there is some level of consistencies between the in vitro and in vivo responses to formaldehyde exposure.

The next chemical I will talk about is glutaraldehyde. Glutaraldehyde is a disinfectant, preservative and crosslinking reagent. It reacts with amines and thiol groups to deactivate proteins. NIOSH recommends that an exposure level ceiling of .2 ppm.

Glutaraldehyde is a irritant and causes respiratory sensitization. Non-neoplastic lesions have been found in the nose of the rats exposed to glutaraldehyde. However, there is no evidence of carcinogenicity.

So the aerosols of glutaraldehyde were generated using the cloud system, which is an aerosol generation

system. That position doses of up to 38.6 microgram per centimeter squares can be achieved using a cloud system.

The test doses for acute exposures were narrowed down to 2.2 microgram per centimeter square based on the LDH assay and transepithelial electrical resistance. When we do the real study to look at the toxicity of glutaraldehyde, we will also include another set of toxicity with different mechanisms just to avoid confounding effects from protein crosslinking property of glutaraldehyde.

An exposure to a single dose of glutaraldehyde significantly induced the expression of HMOX-1, suggesting the possible induction of oxidative stress. We screened 27 cytokines and chemokines. We found some of the secretion of IL-8 and TNF alpha, and possibly FGF, G-CSF and IP-10 were modulated by a single exposure to glutaraldehyde. Both IL-8 and TNF alpha have been implicated in the pathogenesis of lung disease.

Then we look at the response of ciliated cells. Similar to ortho-phthaldehyde, which is a replacement for glutaraldehyde, glutaraldehyde is also a potent cilia static agent. Besides freezing a moment of ciliated cells, it also decreases the expression of CDC20B, which is a key protein involved in the central biogenesis. The expression of the ciliary structure protein however was not affected

after a single exposure. The extracellular secretion and intracellular expression of the mucin proteins were minimally affected by a single exposure to glutaraldehyde.

So here are the major findings of the very preliminary study on the glutaraldehyde. This is the first culture exposure we have done with glutaraldehyde. Mechanistically, I think the glutaraldehyde responded similarly to ortho-phthaldehyde. It will be of great interest to compare the responses between these two chemicals.

And considerably, risk assessments on new sterilant can also be conducted relative to the approved sterilant using similar approaches. We think such exercises may increase the efficiency of processing future medical device pre-market submissions.

The last topic I want to talk about is the new proposal. This is a continuation of E2200. There are always concerns about donor to donor variability when it comes to research conducted in primary cell based in vitro cultures. This new research protocol will explore the interindividual and sex-specific variability by evaluating response to ortho-phthaldehyde using cultures derived from different donors.

There are two objectives for the study. Objective number 1 is to establish a compendium of genomics

and transcriptome profiles of ALI cultures derived from 10 healthy male and 10 healthy female Caucasian donors.

Objective number 2 is to evaluate the toxic response to ortho-phthaldehyde using repetitive treatment protocol. For this study, instead of using the cloud system for aerosol generation, we employ a continuous aerosol generation and exposure system to mimic human inhalation exposure durations.

In this continuous aerosol generation system, the chemical solution is continuously pumped into the nebulizer, which is this little bottle here, and nebulized. Moistures in the liquid aerosols that are removed in the cold trap. Dry aerosols are delivered to the exposure system.

For exposing the cultures, a exposure module equipped with 48 positions will be used to improve the throughput of the study by testing two donors at the same time. So for the experimental design to expose the cells, we are planning to test one female donor and one male donor at the same time as a pair.

The experimental approaches are pretty straightforward. First, we will validate the aerosol generation and exposure system to determine the doses that can be achieved using this new exposure system.

The preliminary dose range finding studies were then conducted using the repetitive exposure regimen. And tissue responses will be monitored using select endpoints from our assay panel. So for the preliminary dose range finding studies, we will try to accomplish three goals.

First, the exposure doses should be relevant to human exposure levels. And second, longer daily exposure duration is desirable because OPA poses occupational hazards. And by exposing the cultures for longer durations every day, we expect to decrease the total number of exposures.

Once the doses and treatment regimens are determined, we will start to evaluate and compare the donor variability to OPA exposures. Our collaborator at CDRH will conduct a risk assessment on OPA using these experimentally determined uncertainty factors. And so that is all I have. I quickly went through the slides. Now, feel free to ask questions if you have any.

Agenda Item: Discussion

DR. DA COSTA: I have had a number of reasonably long discussions with Xuefei about the work that she was doing. I must say that I was always impressed. No matter which question I asked where I thought, I am going to get you there, trying to challenge the system. I think the

systems need to be challenged. And Xuefei has always been a go-to to answer the questions.

I think that the value of the work that Xuefei has presented to you today goes beyond the incorporation of the final cell system. There is a lot of work that she put and effort that she put towards making sure that she understood and characterized the exposures.

She faced some really strange, from a chemical standpoint, kind of foreseeable, but strange events like polymerization of the aldehydes in the delivery system. I am afraid that very few other ventures that are delving into this realm testing paradigms are going through the care that Xuefei put into characterizing exposures. That is a really crucial element.

So anyway, congratulations for that. I think that in advancing to these next stages, we are also addressing issues that are very real about what constitutes an end in new testing paradigms, et cetera. But I would like to hear from you, what your thoughts are on Xuefei's work and proposal. This includes NIOSH, of course.

DR. CAO: Actually, the formaldehyde paper generation is the most challenge part for the formaldehyde study. We were not able to stable generate formaldehyde vapors because of the parameters were not determined correctly.

When you work with the vapor generation system, the manufacturer usually asks you to start with an Excel sheet with the calculations. But it is only for neat chemicals, not for formaldehyde. It has different components. So there is a lot of trial and error that you have to go through in order to get the pumping speed correct.

I can talk about tomorrow, what will happen if the pumping speed is too high. This is something we have found out accidentally. It is not always more is better. For formaldehyde, you have to be in the right range. That is the tricky point. But we are glad. Our team members really spent a lot of time figuring out this problem.

DR. COYLE: I am Jayme from NIOSH. I wanted to raise the question, and I think it would probably be good to have a broader discussion with more individuals. You have a lot of the testing paradigms. You are doing LDH and cytokines and what have you.

I have always been of interest in aligning these with more standardized test modalities, maybe those that are either put forth by NIST or international agreements on various either in vivo or in vitro testing paradigms. Is that kind of what you guys are going towards as well? Is that of interest? What are your thoughts on that? I would

like to broaden it out to I guess a general discussion, if that is possible.

DR. CAO: Yes. Eventually, we are interested in standardizing all the procedures related to what we are doing. What we are doing now is more like exploratory, specific to inflammatory response. There are a lot of caveats. Goncalo and I talk about inflammatory responses, whether you have additional immune cells or if you have additional other cell types, that may alter the immune response.

So what we are doing now is the simplest version of the model just to look at. I think the only affirmative conclusion we can draw from the cytokine screening is there is an initiation of inflammatory response. But how are they related to the immune responses. Say in the humans, we don't know yet. There is an indicator, but it may not be affirmative answer to address what kind of disease consequence, this kind of immune activation will cause.

DR. ZHOU: I believe that you have already data, but I want to know how can you measure the deposit dose?

DR. CAO: We have a fantastic chemist. Unfortunately, he is not going to work on these projects. He left the chemistry group.

So for measuring the deposition doses, I can go into great details. That is going to take at least 40

minutes or one hour to talk about. So for the deposition doses, there were tricks to get the doses correct. And these were the tricks, the manufacturer didn't tell us. And it is not because they don't want to tell us. They didn't know. The way they validate a system is different than how we validate the system.

So when we just directly borrowed their method, we found there is a huge variation for deposition doses at different positions in the cloud system. And it took us three months to figure out how to generate aerosols consistently within the cloud system. So I can share with you all the tricks we use for that matter.

DR. RUO: I just want to say that I am very supportive of this work. At this point, the one chemical here that you mentioned OPA would be of use, regulatory use for CDRH. OPA is a high-level stress disinfectant. So medical devices that can not be heat sterilized use chemicals such as PA.

And there are fumes that then come off from these devices. The FDA recommends certain limits of exposure to the fumes. And OPA still doesn't have that recommendation. I believe this work, especially the comparison. So I am very strongly supporting the work on OPA and glutaraldehyde side by side because that is what companies are trying to do right now, replace glutaraldehyde with OPA.

And there is an exposure limit set and recommended by FDA for glutaraldehyde. But this work, I think, the work that she is proposing, I think it will be great to give us more ammunition to set a good exposure limit for OPAs.

And the one thing that we would like to do, this is still a little bit of a proof of concept because there are certain medical devices that I think will have similar issues where there are fumes and inhalation exposure. So we would like to take this tool through the MDDT processing CDRH. MDDT means medical device developmental tool, which means that we would like to qualify the ALI model system for medical device applications. It is not a full-blown validation, it is just a more abbreviated qualification to address medical device circumstances and medical devices where we have identified this technique could generate data that can be submitted in premarket approvals.

So I think the data that she has generated is great to help us go through the MDDT process. And actually, the one thing that is going to be key for that is that we have to sort of figure out what are acceptable levels when companies in the future do ALI testing and report to us data. We would like to have some sort of acceptable levels in terms of the quality of the data.

So one source of variability is going to be donor to donor variation. I think the work that she is proposing is going to be key to generating the data to get us through the MDDT process and getting approval.

But there is already at least one company that has submitted data to FDA for a medical device. They submitted an ALI. They submitted data generated with an ALI model. We are expecting more such data. But we would like to have a good handle on acceptance levels. So this proof of concept to me is very valuable. I am very strongly in support of it.

DR. DA COSTA: Thank you.

DR. WALKER: I wanted to make sure that everyone realizes that both the formaldehyde, glutaraldehyde and ortho-phthalaldehyde, there are NTP inhalation studies in rodents already conducted. Doing that comparison will have not only the chemical comparison, but will have it by different systems, traditional, full-blown inhalation systems. I want to make sure that you have that for the ortho-phthalaldehyde. The NTP report is out and came out in the last year.

DR. RUO: I am aware of that. In the future, if we have a new mixture, let's say somebody tries to sell us a mixture of glutaraldehyde and formaldehyde, then we can actually do the same comparison. What is the OPA versus

the new mixture look like in this model? We don't have to do an animal study for it.

DR. WALKER: That was kind of the whole reason why we wanted to go down this route because there was a nomination for dihydroxyacetone. When that originally came to us, it was like, oh, my, because standing for full-blown inhalation system is lots of time and lots of dollars just to even get it going from a regulatory point of view. So if we can do that more quickly.

DR. SLIKKER: Xuefei, a very nice presentation. You hit two issues I think in your newer study that you are proposing that is really essential to moving in vitro methodologies forward using human cells. And that is the variability that has already been mentioned, learning about that by doing at least 10 samples. And also the male-female differences.

These are things that we always ask about and think about wanting to have. But you area actually generating the data, so we can see what impact they have. So I really appreciate that for moving the field forward.

DR. HEFLICH: Maybe I can say something that sort of clarifies questions people may have about the system with the sort of enthusiasm for micro physiological systems. The exposure and the quantification of exposure in this is quite unique in the way it is carried out. And

the way this system works is an open system. It makes it amenable to that.

I am not aware of a closed system that, although people are trying to make cigarette smoke exposures in MPS systems and things like that, that really comes close to what you can do as far as measuring a dose to the cells. Which is important for understanding how what you see here relates to what people are going to expose to in vivo. I think even though this is an old model, it has real value for inhalation toxicology.

DR. DA COSTA: So if there are no further questions, then we will actually break now. I apologize for wanting to break on your presentation.

All right. So we will reconvene at 2:15 for nano plastics. Thank you.

(Brief recess)

Agenda Item: Nanoplastics, Update

DR. DA COSTA: Welcome back everyone. So we're going to the penultimate presentation of the day. Dr. Patri will give an update on the state of the art on micro and nanoplastics, I'm sure you've been following all of these affairs on the news. Anil is going to bring some sense of how we stand and perhaps enable some discussion of where we should be going.

DR. PATRI: So, I would like to update on micro nanoplastics. I gave a presentation last year, Nigel asked me to provide an update, so I've focused more on the scientific studies that were done until last year, and Nigel asked me to provide an additional update.

So I wear two hats, one is as Director of Nanocore at NCTR, and as the Chair of the Nanotechnology Task Force in OCS. So that enables me to interact with other agencies, and some of this is coming from those discussions. So these discussions about micro nanoplastics started in 2016, the Global Summit on Regulatory Science on Nanotechnology Products and Applications.

And since then there has been a lot of news, you must have seen if you drink tea with the teabags recently there is a publication on plastics leaching out of the teabags, and then microplastics in water if you are drinking out of those plastic bottles.

The overview is mainly this being a global problem and the progress since last year. The Interagency Interest Group on Nanoplastics that we formed the last month actually in September, we had a Global Summit on Regulatory Science on Nanotechnology and Nanoplastics, I'll give you a brief update on that. There was a congressional inquiry back in June of this year on nanoplastics that came through, again I'll update on that.

And many key knowledge gaps and strategic collaborations, the unique position we have through this interagency agreement to collaboratively work with other agencies to minimize redundancies, improve efficiencies through coordination and collaboration, because this is such a big problem.

And what potential studies can be undertaken under this TSSRC. And this is where I would like to open the discussion, because this is just not one person, one group, one center, it's a cross-center issue, and I would like you all to propose the kind of studies that we could do here.

So that we are all on the same page, you have seen this slide last year, what are micro and nanoplastics. They can be engineered particles or generated from bulk plastics through degradation. No standard definition exists on what microplastics and nanoplastics are, but anywhere between five millimeters to 100 nanometers are generally considered as microplastics, and one to 100 nanometers traditionally the nanotechnology area of the size range that concerns nanoplastics.

They by no means are nice beautiful spherical structures, they can have different shapes, sizes, they can be fibers, spheroids, pellets, flakes, and beads. They can be primary particles made of commercially in micron size,

those that you see in soaps and detergents and things like that, or the secondary particles from degradation of bulk or primary particles.

Needless to say this is a global problem, more than 300 million tons of plastics are produced with 50 million tons of plastics in the ocean, most of them coming from rivers. The debris in aquatic environment degrade into micro and nanoplastics, leading to biopersistence, bioaccumulation, and potential toxicity issues.

A wide variety of plastic sources exist, and again there is a list, again polymers are known for a long time, and they're used in a lot of products, and they eventually degrade into these micro nanoplastics. It could be within FDA< it could be in devices, personal care products, diagnostics and electronics that are relevant for FDA.

Compared to microplastics, nanoplastics have significantly higher surface area, so they can have more chemicals bond to them, and they may include polynuclear aromatic hydrocarbons, pesticides or persistent organic pollutants resulting in potential hazard and exposure.

Human exposure can occur through inhalation, ingestion, and dermal routes. And significant knowledge gap exists. Multiple studies, here in US and across the globe

in Europe, they did a lot of literature search and then came up with significant knowledge gaps.

So the studies that are done are not thoroughly done, and so you can't really conclude anything. Some of them are done based on just using polystyrene, and because they're commercially available with a fluorescent label, most of them are not applicable. And when it comes to nanoplastics again there is a dearth of information, and we don't even know hazard and risk assessment without understanding what kind of compilations and how much quantity we are exposed to.

So quickly, coming to the Inter-agency Nanoplastics Interest Group, this was stood up around a year ago, again based on discussions at the Global Summit in 2016, there are news briefs coming out, you probably have seen CNN documentary and other documentaries that come out for micro and nanoplastics.

And we had a discussion under the USEU Communities of Research, and this is a collaboration between the US and European Union through the National Nanotechnology Initiative, and the topic of the discussion and characterization was micro and nanoplastics, and it became a larger discussion, and then we started this interest group through the NNI.

And the first conference call a year ago in September 2018 had around 10 members, Nigel of course is one of the initial discussions we had with, and he's interested in this topic. Currently we have more than 60 members from more than 20 agencies across the US government, and all of them interested in this topic. So this is an N.

Trying to compile data on who is doing what, who is interested in what, what kind of resources they have, what kind of studies they can do so that we don't have to repeat the study some other agency is doing, and then we can minimize redundancies in the government spending on these topics.

So some of the thrust areas include collection of nanoplastics, there are agencies such as National Oceanic and Atmospheric Administration or Navy, they can collect the water or organisms from different parts of the globe in the oceans and rivers, but then they may not have resources to characterize, isolate, quantify and characterize what kind of plastics are present. So there are groups within these agencies with resources to do the characterization, so that's the first area. Again, the third group is on exposure, hazard, and risk assessment that I can think of. And the agencies are interested, including FDA.

And the mitigation. We know this is a global problem, this is only going to increase. And you cannot completely avoid plastics in our multiple countries. Let's say they ban single use plastics, a lot of single use plastics banned in Europe. Plastic straws are banned in some places, micro beads are banned here in the US and abroad. But these are only a small component of plastics. We cannot live without plastics for sure.

And so there are agencies funding proposals looking into alternatives, to have the same kind of qualities but maybe then easily biodegradable or recyclable, or they call it upcycling. So I'll show some examples of that, there are a lot of agencies conducting the studies and also trying to fund studies that have long-term sustainability.

So EPA got a head start early on in 2014. They had an expert panel discussion on possible human health risks from microplastics in the marine environment. That is the first bullet, first study there, first workshop they had. And since then they had multiple workshops, instead of the science white paper somebody on literature and plastic pollution in aquatic dependent wildlife and Microplastics Expert Workshop, Trash Free Waters Initiative with the EPA. But all these, if you read through them, and I'm not going

to go through each of them, the general conclusion you will see from each of these is that the information is limited.

Certainly, it is a concern, the current state of the science does not allow an assessment of possible human health risks from ingestion of seafood contaminated with microplastics to that from persistent bioaccumulative and toxic chemicals. They call them PBTs. And certainly, further research is needed to gain knowledge in the extent of which plastics transfer contaminants to organisms. And so again there is a website that you can go to at the EPA.

The NOAA, there is a marine debris program you probably know under the National Oceanic Advisory Administration. It's an externally funded program, they have a limited budget, they fund proposals, they're also looking at micro and nanoplastics. The USDA is looking at micro and nanoplastics. We had presentations from Honda Chen from USDA at the Global Summit. So NIH again is funding some of the work, as well as NSF. NSF has started funding proposals in this space.

So DOE, Department of Energy, is funding basic energy sciences on upcycling of plastics. The main problem is that these plastics, single use plastics are very inexpensive, that's why they are single use, you can use and throw. They're very cheap to make, but very expensive to recycle, and that's a fundamental problem. If you take

polyethylene, recycling is a big problem. So the DOE is coming up with funding ideas where they call it upcycling.

So you take something such as a single use polyethylene, and then using a catalyst you can make a high value product, and there is an example, a recent publication this year on using platinum nanoparticle catalysis to take polyethylene into high quality liquid products. And so they are really funding research in this area, basic research.

So the idea with upcycling is to deconstruct these plastics or polymers and reconstruct and functionalize. So the polyethylenes are all hydrocarbons, and then for many of these other products if you have butanol or any of these gasses you need to functionalize them, so there are funding programs to look at the deconstruct, reconstruct, and functionalize. So that's where they think the future is. So again there are websites available, chemical upcycling of polymers. This is a DOE basic energy sciences program.

So if you look at the current state, only 20 percent are recycled, 25 percent are incinerated of these plastics, and 55 percent are landfilled, and so they're going to stay there forever.

I was talking to the State Department rep the other day, and he said most of these plastics actually come

from the Asia and Pacific region, so the state department is now working with again that area, the G7 countries, and so they wanted to expand that research and fund some programs in that area. And they have proposals that are coming up in December.

So again, I'll come back towards the end about the interagency interest group, and then maybe introduce a few more agencies about what their interest is. Certainly at FDA we discussed at the Nanotechnology Task Force, we discussed with Suzy and CFSAN colleagues from Seafood Safety and ORS, some of them I'm pretty sure are online, and to figure out what can we do, what kind of research can we do and strategies that we can work together on.

Quickly coming to the congressional enquiry, this came up in June of this year, Senator Tom Udall and Congressman Alan Lowenthal sent a letter to the President urging to develop a coordinated interagency research response plan. Luckily by then we had the interagency interest group on nanoplastics to address the significant threat that mismanaged plastic waste poses to human health.

The strategy to limit single use plastics, either banning of any such thing to reduce plastic pollution. Again, they confirm plastic recycling is not a realistic solution. Mainly it is economical, it takes more money to recycle plastic.

And the things that I mentioned before, the micro nanoplastics in the aquatic environment, and then the threat they pose to the marine life, and also they absorb chemicals. So they say that well-coordinated and well-funded interagency research plan coupled with robust investments in a response program is essential to address plastic pollution crisis at both human and ocean conservation level.

So they recommended that the departments and agencies across the federal government develop a coordinated interagency research and response plan to address this growing problem.

So this was then assigned, this I guess came through the OSTP, Office of Science and Technology Policy, assigned to the NIH out of the HHS and NIH responded, and of course it went to Nigel at NIEHS, and he knows what was happening at the interagency interest group, and so he could document the response on behalf of Francis Collins. He's also the co-chair of the National Science and Technology Council under OSTP, apart from being the NIH director.

So his response included to the senators' queries to commit to ensuring that the issues raised are given appropriate interagency attention and cooperation, and coordination of federal agencies on nanoplastics through

the NEHI working group. So I serve on the NEHI working group on behalf of FDA, which is under the NSTC's NSET, which is Nanoscale Science Engineering and Technology subcommittee.

And the response includes the interagency agreement between NIEHS and NCTR/FDA, and the discussions we have had on nanoplastics and microplastics, the interest, activities, resources and plans of each agency, including interagency collaboration. And then the approaches to identify and measure nanoplastics in environment and products, including food.

And so this is a discussion we started back in as I mentioned GSRS16, and then this is followed through, again those of you who don't know, the global summit on regulatory sciences, part of the Global Coalition on Regulatory Science that Dr. Bill Slicker co-chairs. And so every three years we bring the nanotechnology topic back.

So the GSRS19 less than two months ago was on nanotechnology and nanoplastics. This was proposed back in GSRS18, which was held in Beijing and China. So by then there was a lot of interest in Europe, so the GRC Joint Research Center under the European Commission, they were willing to host this meeting, and they proposed that the nanoplastics be a plenary topic of interest. So since then

we've worked through and we had a very successful meeting, I'm going to talk about that briefly.

And also, the NIH is interested in advancing the scientific understanding of plastics and how they may be affecting human health. So grants may be coming through NIEHS, certainly as I mentioned NSF and other agencies are funding these proposals. So this is a response that came out of the congressional inquiry.

And as I said fortunately there is an interest among the agencies, multiple agencies, and so this group grew big, now it's 60 people, that's the largest group within I should say NSET, within NEHI. And so there is an interest in making this into a Nanotechnology Signature Initiative called NSI under the NSET, NSCC. And so if that happens then there is funding that is associated with that, the agencies have to report funding for that NSI.

So coming back to the Global Summit on Regulatory Science, again Dr. Bill Slicker and Marta Hugas from European Food Safety Authority, they're co-chairs of the Global Coalition. We had the meeting in September in Stresa Italy. It didn't help that it's a beautiful location, it's in the lake country in Italy, it's cohosted by the JRC European Commission. They did a wonderful job, very successful meeting, with 200 people from 34 different countries.

More importantly the attendees came back to me and then said this is not like the regular meeting they attend, regular scientific meeting, this is more focused on regulatory science, mostly regulators, looking at the knowledge gaps that existed, and then how can we work together from a regulatory standpoint.

And so people presented progress and exchange views. Again this is a follow-up to the global summit we had in 2016. So it included topics related to drugs, devices, toxicology, food. The European Food Safety Authority led that discussion, complete panel on food related nanotechnology and consumer products. So all these topics included, with a plenary session on nanoplastics. SO people were not happy, when there were panel sessions they wanted to be at both locations at the same time, because the topics were very interesting, so we made nanoplastics as a plenary session.

And you can see the Scientific Program Committee. I co-chaired the Scientific Co-chair Committee along with Birgit Sokull-Kluettgen from JRC, but then there are all the global coalition member agencies that are part of the scientific program committee, Canadian food inspection agency, EFSA, EMA, Health Canada, again from China the National Institute on Food and Drug Control from China, so it was a wonderful meeting, and all of them stayed until

the very end, which is very rare, and really focused on these topics. So I would just focus on the nanoplastics, which is what I was asked to present.

So we had a lot of presentations. We had only one I should say from US, from EPA, Souhail presenting on detecting nanoplastics in the environment, adapting existing methods from nanotechnology. But Nanna Hartmann from Denmark presented on the risks of nanoplastics to humans and the environment, the state of the knowledge, and highlights from the SAPEA Report. So this is a report that came out of a big committee that looked at everything that is available, and that will show you one slide about that, but the nanoplastics in the real world, again this is research presentation and environmental fate from Julian.

He presented about using pyrolysis GCMS. SO you can take pyrolysis GCMS and quantify the kind of plastics that are present, but then there is another technique called TGA IR-GCMS, which is a hyphenated technique, to identify using infrared spectroscopy and GCMS to quantify the kind of plastics, to separate and quantify.

That presentation was given by Iseult Lynch, he's from University of Birmingham. Again, I'll briefly talk about what she presented. But also the European Commission perspective, Sylvan Bintein from ECHA also brought their restriction of intentionally added micro and nanoplastics.

They have restriction from ECHA on the use of micro and nanoplastics in Europe, and also on the risk assessment from BFR. SO we had really nice presentations on multiple different topics within the micro and nanoplastics area.

So one thing is most of the topics that were presented were on microplastics, because there was very little known about nanoplastics. And that is the bottleneck. So we don't really know a whole lot, and all of them keep saying that, but a little is known about nanoplastics.

So the SAPEA Report is to provide a scientific perspective on the current knowledge about the impact of NMPs, NMPs are nano and microplastics, in nature and society. Nanna Hartman presented the summary of the findings on microplastics.

So when it came to nanoplastics, again there is not much information available on the characterization, the hazard exposure, risk assessment. So if you go through all these, the final conclusions kind of say that there is not much known, and it is not feasible to distinguish between NMPs and large nanoplastics when reviewing and defining regulations.

Not only that, when you go through the very few studies that are done thoroughly, and I have gone through a lot of literature in the last two years, to kind of figure

out whether we can replicate those, whether we can use those techniques to identify and quantify, most of the studies are based on commercially available polymers, such as polystyrene.

Very few actually get into the real world situation, taking a fish out of the ocean, isolating those, and then quantifying and identifying the very few, even if they did those kinds of studies, even those are spotty, they are not thoroughly done, because of lack of methods and procedures, and Canadian Food Inspection Agency is looking into some of those methods.

So Iseult Lynch's presentation was interesting. They are doing a project, it's called 100 Plastic Rivers Project. So as you know the plastics end up in the ocean from the rivers, any person out there. So they concentrated, and this is an EU funded program on getting these waters from 100 different rivers, and that's why they call it 100 Plastic Rivers Project in UK. The Project Lead is Professor Stefan Krause. This is to coordinate the first systematic and global analysis of microplastics in freshwater ecosystems.

So they wanted to develop a database, which is great, because then we don't have to develop a database. If someone is developing a database and it is freely and openly available, any data that anyone develops or comes

up, as long as it is curated we know exactly where it is coming from, it is a good dataset, it can go into this database so that people can then compare their product with a database and then figure out where it is coming from or what the composition is.

So they started taking anything in their labs and their homes that contained plastics, this is a first starting point, and then chopping them into pieces and running TGA-IR-GCMS, get the data, get them into the database. So they started this database with different products, and then identifying which product and what is the composition.

So if you end up let's say isolating from some seafood, then you can kind of figure out what the composition is by comparing with this database. I thought it's an excellent tool. And then again they are trying to model microplastic fate and transport, and then identify impacts on freshwater ecosystems. The TGA-IR-GCMS is something fortunately we could get at the end of the last fiscal year with the end of the year funding, and so we have that available at the nanocore.

So this is a link to the database here. What we discussed at the global summit is if someone is developing a database then all of us work together to provide data to them so that the data is at one place and not necessarily

at multiple places. It's not easy to maintain them anyway, so someone is willing to do it, you enable them to maintain the database.

Again there are a lot of presentations there. I can provide more information later on, but I just want to give you a flavor of what happened at the Global Summit. EFSA is really interested in micro nano plastics, they had a panel a few years ago, and they have another meeting in June specifically on micro and nanoplastics in their facility in Italy at EFSA.

So as I mentioned the inter-agency coordination and collaboration came through. The NEHI working group, we have a lot of my colleagues from FDA that are a part of that, from NIH, from EPA, again there are 20 agencies that are part of that, as of today there are 65 members in that interest group.

So when I looked at the portfolio of what people wanted to do, we started with just an excel sheet to kind of ask what is their interest within their agencies, what kind of resources they have, what kind of instrumentation they have. So some people don't have any resources or instrumentation, but they have the capability to collect samples. We don't have that capability, they have that capability. So we can collect the samples.

So I kind of came up with this diagram to look at, okay, sample collection, there are some agencies that are interested. Develop methods, analysis, there are some agencies that have capabilities and interest to do biological studies in vitro, in vivo studies, exposure studies. Some have very good modelers that can do hazard, the risk assessment. Others have interest in databases. And of course these other policies that we all develop is based on solid science that comes out of that kind of work. Some agencies such as NSF/NIH and DOD are interested in research funding. We can all do gap analysis and then modeling.

Standards, NIST is interested in standards, they already started asking can we get micro nanoplastics reference material standards. The problem is okay, what kind of standard. They already have polystyrene standards, 100 nanometer of polystyrene available, but that's not really the micro nanoplastics that we talk about, even though it can be part of that, these are the kind of questions from the developed standards, tell me the compilation, tell me the surface, they get into the details of what the measure are for the particular standard. And I mentioned about upcycling, this is a significant interest for DOE.

So I put together, USGS, EPA, DOD, NOAA, they have the capabilities to do sample collection. We have

certainly capabilities at FDA to do characterization, the same thing with NIST and EPA and NIH. So these other agencies have interest in exposure hazard risk assessment, and some of the other agencies are interested in funding. So that way we can come up with eventually a coordinated effort of this global problem. We don't have to do everything, we can probably contribute a small piece of the big puzzle, both working with other agencies within US but also internationally.

So coordination domestic and international, minimize redundancies and enhance collaborations. So in that context I certainly feel, and I know Nigel has too, that this TSSRC, this interagency agreement and collaboration can play a significant role in addressing this global challenge. We can certainly look into the methods, development, in vitro and in vivo studies.

And Suzy mentioned earlier about a pig study, and we can discuss that. There is ICCVAM NanoWorking group, and we are part of that. Alternate models, in vitro models. Certainly we can work with NIEHS grant support teams from academia to look at what they can do so that appropriate funding can be provided by the agencies based on the gap analysis that we come up with.

So these are the key knowledge gaps, and I can tell you the nanoplastics information is limited. There is

information available on microplastics, but not conclusive information. If you look at let's say some publications where they tell you there are seven microplastic particles per fish, we don't really know what the composition is, what else bonds to them.

So methods development should include the isolation, detection, identification, characterization, and quantization. A lot of these studies, they just characterize from a qualitative standpoint to see I can see 10 micron sized particles. Not many on nano.

But then the quantitative aspect is sketchy. If you want to digest the whole seafood or fish and then isolate the plastics and then figure out, those are the kind of ways that we can get into without compromising the integrity of the micro and nanoplastics. If you digest completely with acid you are destroying everything, but then there may be methods that CFIA is working on, and we may have some here at FDA on enzymatic degradation methods to keep the polymers intact but digest the tissues.

But also, a lot of these studies and reports talk about not only just the micro and nanoplastics, but what is bonded to them. And that information is limited. Chemical simple and complex matrices quantitation.

We could do robust, reproducible in vitro studies, some through NTP, we are working on standards

development through ICCVAM and others. Again hazard exposure and risk assessment of nanoplastics. These are knowledge gaps. So we cannot do everything anyway, but I will open up for discussion on what kind of studies are relevant, and those on the phone and in the room can chime in.

But before that I would just like to showcase on some of the upcoming activities. There are a lot of activities suddenly, and since I am leading this interest group I get emails almost every day. I just got an email sitting there about a meeting in France in December that we should consider, this came from the state department, of course we can go within a month of here for an international meeting, but this apparently is of interest to the State Department, and then they're working with France to organize this meeting.

So as I mentioned about the Nanotechnology Signature Initiative, this is a discussion that is coming up on November 19th at the NSET meeting, I'll call in and then make the case for the NSI. They asked for proposals. So NSI, those of you who are not familiar, if there are multiple agencies interested in a topic then they make that as a priority for a nanotechnology signature initiative. This topic has more agencies than the NEHI working group has, 40 agencies.

So this is on the top of the list for NSI, but they still have to go through the exercise within NSET, and then conference from NSGC where you have the heads of the agencies participate at NSGC, and then call it an NSI. Once it becomes an NSI then we as agencies have a reporting responsibility as to what we are doing and what funding we are providing to the topic within the agency.

Department of State is interested in increasing awareness and collaboration in the Pacific Rim region. SO they asked for proposals, and they have this meeting in Malaysia I think early next year to decide on a future. And then they have some funding, either to do research, mainly for the Asian Pacific region, and this is a proposal they are requesting, and so I just sent out an email about this topic, and then maybe come up with a proposal.

The National Academy of Sciences, there is a workshop that is actually sponsored by NIHS on emerging technologies to advance research and decisions on environmental health effects on microplastics. This is scheduled on January 27 and 28 at the National Academy of Sciences in Washington DC. I was nominated and I am serving on the organizing committee for this. And so we just started this committee, and then hopefully come up with a program within the next month or so. It's a two day program, or a half day at least.

Because of that, and then a lot of the agencies will be there, the NEHI considered just a week ago to have a fed only workshop on nanoplastics through the NEHI. And this is on the 29th at the NNCO facilities. This is in L'Enfant Plaza in Washington DC, so those that come to this meeting can extend their stay.

This is a fed only workshop, no WebEx, all these, if you have been to any of the fed only workshops out of NEHI, we had one on TIO2, titanium oxide, fed only workshop. So similar to that this would be for nanoplastics. And they can only hold 60 people, and if it becomes bigger than, or more people wanted to come to this because it's in Washington, then we have to find an alternate location. Again, if you look at the dates, it's only two or three months away.

American Chemical Society has a meeting on micro and nanoplastics in Philadelphia in the next meeting in March. Souhail Al-Abed from EPA, he's co-chairing this, but he's also co-chairing the Pacifichem in 2020, again the same topic in December in Hawaii next year, December 2020. Those of you who know Pacifichem, it's a micro chemical society in the Pacific region once every four or five years is held in Hawaii, and so they have that meeting, and either they're planning, again there's discussions, the state department can sponsor either in the Pacifichem, or

they're planning a meeting in New Zealand, and that is a possibility for the pacific region.

Acknowledgments, again Nigel, because we have been discussing about this for a long time, but he is also very active providing advice, and NIEHS. To my colleagues from NNCO, NSET, and NEHI, the Nanoplastics Interest Group, I learned a lot through talking to them either at the conference calls that we have or one on one calls, they tell me a lot of things that they are doing. And then they don't have everything, they want collaborations with others. And I think this is a great resource for this to come together on this topic of global importance.

The GCRSR, the Global Coalition that Dr. Bill Slicker co-chairs, and because of that we could actually bring the regulators to one place on nanoplastics, just a month ago, very quickly. And so all these regulatory agencies are involved in that. We all have the common problem, and so if we discuss that then we can figure out some solutions.

NTF has always been supportive of all this work, and also through CFSAN we have members from each center as part of the task force, and then colleagues from CFSAN and also the NCTR-ORA Nanocore. Thank you. And I will bring you back to this topic, key knowledge gaps and what topics we should be working on. Thank you.

Agenda Item: Discussion

DR. FITZPATRICK: So, my first question is I see that Alfonso Lampen had talked about micro and nanoplastics include all of the toxicology. We see, and he may already have presented this, the first question is how much is getting through, how bioavailable it is or what size does it have to be to be bioavailable just through the GI tract, is it different for the different plastic. We recognize that some of the plastics might be plastic wraps from food, so not only is it in food, it might be from some other products. I don't know if he presented that or EPSA already has some idea.

DR. PATRI: so, he is from German Risk Assessment Institute. They did some modeling, at least the information that is known at least from publications, and you have seen those, I presented some of them last year, again in the fish gut, some of the studies in the fish gut. They claim that the microplastics stay in the fish gut and then they don't penetrate, whereas very little information is available on nanoplastics that can certainly penetrate.

There are only a couple of publications, not on the human exposure level but at least in the seafood, that the nanoplastics, there is a publication that came out a few months ago, they did a study where I think they took something like polystyrene, it's not a nanoplastics, but a

polymeric nanoparticle at certain sizes, and looked at the permeations. The smaller nano size permeates through, and then they see that in liver. They also did that study along with a chemical, I think it's BPA if I'm not mistaken, but I'll dig up that reference.

So what they did was just the particle itself, just BPA by itself, and then BPA bonded with a particle, and they show that there is great accumulation of BPA in the liver because it is bonded to the nanoplastics and somehow the bioavailability and transformation is happening. But that is again a model study. So we don't really know, I mean all of them, the final conclusion is we don't know enough.

DR. FITZPATRICK: That is why I was interested in the pig study for people that don't know that many pigs digestive system is close to humans. We do have at CDM a fish aquatic center, I know Tong isn't here anymore, to learn about fish.

We do have, CFSAN does have, is she still here? I think she left. We have Dolphin Island, which is our seafood specialist place down, actually it's on a resort island off the Gulf Coast of Florida, probably a nice place to visit. And so they might be able to do some collections. Our chemists can do some analytical work on measuring some of these. So I think for us the first question is what are

people exposed to, and then once it gets through, what the small size is to get into the cells, I'm sure nano can cross the cell membrane, I'm assuming it can.

And then is it just junk in there? Does it have some action that it can be cleared out? I think we're really interested in, because we recognize this is a big problem, this is a big problem in food, it took a long time to get CFSAN's interest perked unfortunately, but now especially our seafood people are interested and we've formed this CFSAN NNP group, I guess. I think that's our first question. Hopefully we can get some research to start into that, because there's no point in citing what the toxicity is if we don't know if it's there or not.

DR. BELAND: On that chart you had of the agency coordination. Is this to imply that within the federal government we're not doing any biological studies? Go to the next - Maybe it's just the way it's constructed --

DR. PATRI: This is not to say, no this is not specifically with a pointer to say only analysis is done, it's generally. So biological studies exposure assessment, assessment, I just put in NIOSH, EPA, FDA, NIH. Also this -- this is an interest but it's not meant to be comprehensive, it's not necessarily comprehensive. But if I can use a pointer here, so all these biological studies can be a lot, in vitro, in vivo, exposure has a risk

assessment. And I just put that group of agencies interested.

DR. FITZPATRICK: So is it possible to get funding to do this kind of study at NCTR? I mean I don't think we're going to do it anywhere else.

DR. WALKER: We've already been funding Anil to do this. So starting three years ago, after the GSRS, we discussed this, it's like this is classic, where the interagency agreement sits, is this something that should become a high priority, if so how, it's an intersection of like every dollar actually increases value of information, because as opposed to incrementally decreasing additional information. And we sat down and it's like what are the next steps. You're not going to jump in and do tox studies because that's kind of what, I hate to say, that's what academics will do. The question is where is it, hang on, how do you even measure this. So we spent time chatting about what are the methods.

So actually Anil presented last year a few review that we funded them to do of how do you even distinguish between nano versus micro. And not even we did some EM work and we can see some, you'll see papers, you can guarantee you'll see the papers of hey we've identified nanomaterial plastics in you name the species, you'll see the papers. It

won't be quantitative, you won't be able to use it, it won't address real world samples.

And so it's like strategically let's figure out how to measure, let's start getting folks coordinated, let's start understanding what people are actually doing, strategy, gap analysis, let's identify the gaps before we actually start jumping in. I'm going to give Anil kudos for really kick starting and bringing them to the table, rather than just going in like headless chickens running after it. We've started that strategic approach of getting people talking, and this GSRS and the way that we are with it now is testimony to having a very methodical approach to this.

DR. FITZPATRICK: (off mic) -- unfortunately we don't find out about this GSRS until it was too late to tell.

DR. WALKER: I think Anil presented that that was in the cards back in May.

DR. FITZPATRICK: That is what we are interested in, knowing it's an issue in food, it will be an issue in food if it's not in there already. We've already been asked about bottled water.

DR. WALKER: We are totally aligned with this is an important area where we need to get folks coordinated.

DR. FITZPATRICK: If NIHS takes the lead on it that's great, or the nanocore. We just want to make sure

that we're part of it because ultimately we're the ones that are going to have to --

DR. PATRI: So expand the current funding to the nanocore. It's difficult to recruit, I can tell you, all the division directors are in the same boat. Difficult to find the expertise and the right people to do the work. But given that limitation, we have the equipment, instrumentation, infrastructure supported by NCTR for a long time, this is amazing that we could do that work. The other possibility is some of the, we can't bring people from outside, but then certainly within the FDA, within the government can come to the labs and we can do some of the work that way. But of course, additional funding doesn't hurt to focus on the micro and nanoplastics. FDA, we are focused on standards development, I presented that six months ago.

DR. CERNIGLIA: (off mic) - pathogenic microorganisms in there, so that's another source of risk to humans that needs to be considered in a total risk assessment. But the other point that I'd like to make in terms of research ideas and all, in my own particular group in collaboration with the GMT, Marley Asovado(phonetic) and Javier Rovalo(phonetic) put together a proposal, collaborators on microplastics (indiscernible audio) toxicological endpoints, microbiome endpoints, using the

cake(?) model, nobiotic cake with Ohio State University. And the key to this particular study, and this is what I mentioned earlier about when you're designing studies looking at that tipping point and window, this is a neonatal pig model that they're using, and then in this notobiotic they're germ free, but then they're adding infant human microbiota to that animal, somewhat more like a mammalian model. So it's very good HCE grant, so we can see what happens.

But I wanted to highlight the fact that we recognize this whole issue in microplastics, nanoplastics, and so with that there is proposed, and one of the strongpoints of (indiscernible - audio) we are working together across center as well with Ohio State University which has nobiotic animals facility, the pig model. And also there has been studies actually on, because of the inflammatory response of particulates in the GI tract, like these plastics, especially polystyrenes, they went from 50 to 300 cutoff in exposure size. So that kind of work is also being done.

DR. PATRI: So EPA actually presented some study, Kaho(phonetic) from EPA at the Global Summit, which I thought was very interesting. So they were trying to figure out how to measure these microplastics. So they found more plastics in the air in the lab in the very clean controlled

environment. So they had some kind of public news brief or someone, they said this is what we found.

So the people who were asking questions, they suddenly forgot about the study, they were asking about what you're breathing, what is in the environment. So the control studies, what brings the EPA, the main focus of the presentation is the controls have plastics. This is not dissimilar to the study that was done at NCTR, to actually clean plastic at EPA before you can do a study on BPAs.

PARTICIPANT: I have a quick question. It's good that we have a lot of (indiscernible) but is any information of concern about the path you may be getting with the treatment incinerated the particle and then generated a lot of dust. Actually there's a concern on that because of occupational related, and I think it's a concern for EPA, because dust can be contamination to the soil and can go into the river, can go into the water, and people can drink it. So I don't know if you have some informational concern or plan.

DR. PATRI: The only information that we are gathering is from the agencies, including NIOSH for example, and then any publications that are in the public domain. So I didn't see so far, any kind of that information on when you incinerate these plastics, whether

some other micro nanoplastics particles are released in the environment.

But certainly there are publications that show in the architect you have microplastics. With nothing in there, they had an umbrella, and then they collected microplastics particles. This is a publication a few months ago. So it's in the air. The bottom line is microplastics and nanoplastics are in the air that we breathe in this room. In this plastic bottle there are up to 1000 micron particles per liter, depending on different plastic bottles, that information. Thank you.

DR. BELAND: So, you've talked about the government perspective. What does industry think about all of this?

DR. PATRI: At least what I am doing so far is with interagency, we didn't really get into the industry. So hopefully with the NAS workshop, the National Academies workshop, so there they wanted to bring not just academics but government, industry, and then even the public interest groups as part of that meeting, and maybe something comes up. These are, in some ways they're polymers, and we didn't really talk to industry yet.

DR. FITZPATRICK: When I brought this topic up to HESI(?) because they were looking for emerging topics, they said oh no we can't do this, we're talking with the

American Chemical Council, we're talking with Canadians, nobody wants to talk to you guys, but maybe we should be. So I think the industry is interested, they're just not really letting on as much.

DR. DA COSTA: Thank you Anil. So we're running a little bit behind, but I think it is worth it. So the next update is going to be given by Dr. Fang in his work on PEGylated biologics.

Agenda Item: PEGylated Biologics, Update

DR. FANG: Hi everyone, I am going to give you an update on toxicity of high molecular weight polyethylene glycols. PEGylation is a process of both covalently and non-covalently bonding of a PEG polymer to another molecule, normally a drug or therapeutic protein/peptide. PEGylation can increase the bioavailability of the drug, reduce the frequency of the administration, it increases the half-life of the drug and optimizes the pharmacokinetics and masks the antigenic determinants of the proteins and the peptides.

PEG is a polymer of ethylene oxide, and PEG weighs five to 60 kDa in general used in PEGylated biopharmaceuticals. The PEGs used in PEGylation most of the case are capped with a methyl group at one end in quantities polydispersed in nature. Exposure to PEGs in humans from a single dose of approved PEGylated

biopharmaceuticals ranges from 26 microgram to 176 milligram PEG.

And in pre-clinical studies, several PEGylated biopharmaceuticals have caused PEG accumulation and cellular vacuolization in various tissues, including the choroid plexus. There is a concern that PEG accumulation and the formation of these vacuoles may lead to adverse outcomes for PEGylated biopharmaceuticals used chronically and/or in pediatric populations. There are a data gap on the tissue levels of PEG over time, and the long-term effect of PEG on some tissues, especially the choroid plexus and kidney.

And to address the data gaps identified by CDER and CBER, we propose three studies: a single dose toxicokinetic study, a repeat dose bioaccumulation study, and a toxicity evaluation study. And due to the difficulty to obtain a pure radio labelled methoxy PEGs, we postponed the toxicokinetic and bioaccumulation studies and proceeded with the toxicity evaluation study.

And the toxicity evaluation study work was conducted against regulatory risk(?). The first dosing study in December of 2018, and the last animal was sacrificed in June 2019. There are two moribund animals, one animal has severe skin lesions at a subcutaneous

injection site, and another has a severe degloving injury that tears, and all other animals survived.

The body weight, food consumption, hematology and clinical chemistry, and the histopathology results are being statistically analyzed. The formation of anti-PEG IgM is being examined and requires further validation. This data will be reported in the next TSSRC meeting. And today I will report some data, the formation of anti-PEG IgG, the levels of PEGs in plasma, urine, feces, and CSF, and additional issues with immunohistochemistry were discussed.

And these anti-PEG IgG in plasma, flow cytometry was developed. These rat anti-PEG IgG standard was a gift of Dr. Brofola(phonetic) and was confirmed by the western blot. And as shown in this histogram from the flow cytometry assay the red anti-PEG IgG standard has with a 500 dilution has a much higher flow rate and intensity compared to the background levels observed from a five percent PSA. And the levels of anti-PEG IgG in plasma was examined at the end of each 4 week period. From here we can see the flow rates and intensity among the treatment group and the regular controls, there is no difference. So the data tell us anti-PEG IgG was not found on 24 week treatments.

And also the levels of PEGs in plasma and excreta were examined using SDS page iodine staining assay. As

shown here we can see the PEGs in plasma, urine, and feces were detected in our treatment group but in controls.

And here the levels of PEGs in plasma is a molecular weight dependent increase in both males and females. By contrast in urine there is a molecular weight decrease in the levels of PEG. And also from here we can see there is no admission route difference between SC and IV in plasma and urine. In feces we can do in females we can see 40 kDa and 60 kDa PEG is created more in feces, but this is a result that we couldn't see in males.

We also made an exam based network of PEGs in CSF using the same methods, SDS page and iodine staining assay, and here we can see the PEG was detected in so was CSF from a treatment group but not in regular controls. The data in the parentheses give you how many animals were detected PEG in CSF. So here when you see it they are high molecular weight of 40 KDA and 60 kDa are mostly all the male animals can be detected, but not in females, and this data indicated the PEG was present in CSF.

And also we attempted to develop an immunohistochemistry assay to (indiscernible) the tissue networks of PEG. For this purpose we used western blot to screen more than 10, these anti-PEG antibodies from mouse and rabbit, then passed on this data which was four high

affinity antibodies, three mouse anti-PEG IgGs and one rabbit anti-PEG IgG.

And also this antibody was confirmed by this western blot. When you look here there's polyoxazoline tween-20 and triton-X-100, widely used in western blot and immunohistochemistry assay. And here when you look at the chemical structure, tween-20 and triton-x-100 has the ethylene oxide repeated unit, they are identical to the backbone of mPEG.

So this gradient, when we use the TBST tween-20 in the PBS to wash in the purity of memory, they wipe out all this signal. So that means when we do the immunoassay to detect these PEG networks in tissues or in this plasma, no polyoxazoline detergents included. So using this condition we take these three anti-PEG, three mouse and the one rabbit anti-PEG IgGs for the immunohistochemistry assay.

And for all these three mouse anti-PEG IgG, where a strong mouse base kit standing. It doesn't matter how much we diluted these primary antibody to one to 10,000, we get a very strong non-specific binding. But for the rabbit this antibody got no staining at all. So now I have to try a couple more times to find out. However once (indiscernible) however this PEG is still retained on a section after the extensive washing and incubation test

steps. That is what I am going to test. That is all. And I'm open for questions.

Agenda Item: Discussion

DR. DA COSTA: This is one of those classic examples of a study that seems to be pretty straightforward when you design it, and then when you actually start working on it it's a real challenge. And I must really praise Jia-Long for how he was able to tackle the issues, one of them for example how to even quantify PEG in the dosing formulations.

And if you go across the literature, everyone talks about using really complex methods that go back to high resolution mass spectrometry, and Jia-Long started out by coming to realize from a fairly old publication that although it's not an unsaturated compound that it actually stains pretty well with iodine, so he was able to tackle that, and which is the method that he used both for formulations and for the gel.

So having said that there's still issues that Jia-Long is working with. If you think about it, the reason why PEG is used to code biologics is precisely to make the biologics less immunogenic. So developing antibodies against something that is not immunogenic is not trivial. Anyway, we'll see how Jia-Long is able to provide us an update next --

DR. SLIKKER: Could you go back to the CSF study? I was really interested in what you were seeing there in the male/female differences.

PARTICIPANT: Could you look at other tissues?

DR. FANG: I tried to develop this immunohistochemistry to look at other tissues. Other tissue is firmly fixed, embedded, so I couldn't use this method. I used this method to detect the PEG in human (indiscernible) treated with PEG.

DR. BELAND: if you notice, the level here is about one one-hundredth of what was in the previous slides. These are very low levels. The intense staining is the standard, and it's the very faint band next to it that is the sample. And if you look at it, if you go back one slide Jia-Long, the histograms, if you look at the scale you can see they're going zero to 1000.

PARTICIPANT: It's really that on males it's closer to the limit of detection.

DR. BELAND: There may be a sex difference, but at the moment let's not take it that way, let's say we're right at the limit of detection. And so what our intention is, Jia-Long mentioned we struggled for a number of months with trying to make triturated material, and every time we did it the stuff blew apart. The plan now is to put on a couple of carbon 14 units, extend the chain just a little,

we're talking about 20, 40, and 60 kD, so we can extend it a bit and still not change the molecular weight very much, and go in with carbon 14 material. So that's the plan.

DR. SLIKKER: So just in general though, were you surprised to see any signal that would be across the blood-brain barrier for this set of compounds?

DR. FANG: So if I could answer the question. The only thing I could tell is that we detect the PEG in CSF. How we get there, I don't know. But when we go to the CSF, based on other data, other CSF or the hemoglobin content in CSF, basically are clean based on hemoglobin contents.

DR. COYLE: I guess the two questions that I have is you're trying to use radioactive, is there any reason why you can't just do a C13 and run it through an LCMS or GCMS or something like that? Am I being overly naïve on this?

DR. DA COSTA: With C13, if you are only going to put a small tail your mass increment would be very minor, and when you consider the isoptic distribution of a 60 kilodalton species it looks like a Gaussian curve, and so you would really not be able to tell it from error. So I keep on saying that all the good compounds have been dealt with, what is left is really weird.

DR. BELAND: I think people have gone away from using radioactive material, but if we can get it made,

which I think we can, for a relatively reasonable cost, it's the easiest way to go through and do these types of studies, because we can go through multiple tissues relatively easily, and so that's what our intention is. Until Anil says otherwise.

DR. PATRI: So one of the standards that we are developing with NTP funding is a PEG quantitation. So all these nanomaterial, because of the same reason, they have PEG floating on the surface, but none of the publications if you look at them, they don't actually measure the coding quantity, which is critical for any biodistribution study, any clinical study.

And so the method that we are using, it's on pristine material obviously, let's say PEGylated gold nanoparticle, and you isolate the polyethylene glycol, we did all the way to 20,000 molecular weight. We didn't test 20,000 or 60,000, and I don't see why it won't work.

Using HPLC charged data cell detector, the count is very sensitive, it's quantitative, linear within the range. But if this is working, and I have a question about that. My point is one could do HPLC CAD if you can isolate polyethylene glycol from blood using HPLC CAD, because it doesn't have any chromophore for CAD or even ELSD for that matter. ELSD is less sensitive, but CAD, and this is a

standard that is going through a valid process at ASTM, so that can be used to quantify.

DR. DA COSTA: That is without the matrix, all the analytics become much simpler.

DR. PATRI: As long as there is a methods development of isolation polyethylene glycol by precipitating the proteins just like any other small molecule into acetonitrile is it soluble in acetonitrite, I don't know. That can be at least tried. But the fundamental question I have is what is moving polyethylene glycol on the electrophoresis. It is not charged, polyethylene glycol is a neutral molecule. I know there are differences in the molecular weight. So if you do STS page there is no amino nitrogen that binds to STS. So the fundamental question is what is moving PEG on a gel.

DR. DA COSTA: It is probably moving like a crown ether. I think that is the same principle. You also would not expect iodine to be able to stain a saturated structure, polyether essentially, and yet it seems to somehow make it a chromophore. And I don't really know exactly how it works, but I think the reason is that it acts like a crown ether. I don't know what the ion is that is contained there and is being cultivated by the ether, but that's probably it. But it's a really good question, I have not thought about that. The fact is that it migrates.

DR. PATRI: The reason I'm asking is we tried. And Nigel knows this, Marina, my previous lab tried this using why are you doing this HPLC methods, I can do LS diagnostics. We bought every known anti-PEG antibody at the time, and none of them gave consistent results, for the same reason, PEG is used to escape that immune system recognition, you don't have good antibodies. And none of them really worked quantitatively, so I'm wondering if there is some antibody that is working.

DR. DA COSTA: In the end we don't know. Ultimately at this stage I would probably put more faith in terms of getting an insight of what may be located where using the radiolabeled component. But it would not be sufficient for autoradiography.

DR. PATRI: I think the method can be validated using HPLC CAD, and then if there is a simpler, easier method, than you know comparing the concentrations and you know they are correct, then you use a simpler method, and not use HPLC CAD.

DR. DA COSTA: Thanks so much. I am not sure if there are any other pressing questions for today, because we're already running a little late. So reminding everyone we're going to have dinner at Eliza's Italian restaurant, I'm hoping that our NIOSH colleagues are going to be able

to join us. So we'll chat about how to get there. Anyway, it's at 6:30. So everyone else is invited to join us.

For those of you interested in further discussions on the LI system. So again we're here tomorrow at 9:00 to 11:00 so that everyone has time to either drive or fly back home. Thank you so much everyone. For those on the phone thank you for connecting. And next meeting in May.

(Whereupon the meeting was adjourned at 4:15 p.m.)